The Function of Volatile Semiochemicals in Host Plant Choice of Ovipositing *Manduca* Moths (Sphingidae)

Dissertation
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„Der Duft der Dinge ist die Sehnsucht,

die sie uns nach sich erwecken."

Christian Morgenstern, 1896

Stufen - Eine Entwicklung in Aphorismen und Tagebuch-Notizen, München: R Piper & Co Verlag, 1922.

The Tangled Bank

Contemplate a tangled bank
Clothed with many kinds of plant
Insects and birds flitting about
Worms crawling through the damp

Reflect that these elaborate
And differently constructed forms
Have been produced by such a simple set
Of ever acting norms

Growth, reproduction and inheritance
Variation to transmit
Natural selection then leading to
Extinction of the less fit

From the war of nature
From famine and from death
Follow the most exalted species
To have ever drawn a breath

There is grandeur in this view of life
And its powers not yet gone
Having been originally breathed
Into a few forms or just one

From as simple a beginning
As could ever be resolved
Endless forms most beautiful
Are continuously evolved.

Michael Eisen, after Charles Darwin, On the Origin of Species

INTRODUCTION

When Charles Darwin imagined his tangled bank in nature, he found it braided by countless complex interactions between all kinds of organisms. The involved species may vary greatly in their origins, states and needs, but they share an elementary language: chemistry. The smell of freshly cut grass, a dog's territory marking or the burning bite of a molested ant are all examples of chemical communication meaning "Help", "hello", "stay out of here", or "let me go". Every organism utilizes chemistry to mediate various interactions (Meinwald and Eisner 2008). The pivotal meaning of chemical signals for insects was felicitously described by Louis Schoonhoven (1996), who greatly contributed to the understanding of host-plant specialization in insects:

> *"To the human eye the plant world is a green world, albeit rich in structures and shapes. Its real diversity is manifested in its chemistry, which encompasses an almost endless gamut of small to medium-sized molecules. Whereas we can only try to fathom its richness and nuances with the aid of sophisticated instrumentation, plant-eating insects can perceive this plethora of substances and employ it to meet their finicky feeding habitats. Clearly, insects live in a chemical world."*

Chemistry is shaping the host plant choice of herbivores. In the late 19th century Ernst Stahl, active at the University of Jena, performed feeding experiments with snail species (Stahl 1888) to examine whether "all that is green is palatable to animals" (Whittaker and Feeny 1971). He found several plant species being chemically defended against herbivores and thus, is considered as one of the pioneers in the field of Chemical Ecology (Hartmann 2008). 125 years later, facilitated by the rapid development of analytical techniques, a plethora of studies focused on chemically mediated insect-host plant interactions. One of the very first insect-plant relationships that got into focus was the adaptation of *Pieris* butterflies (Lepidoptera: Pieridae) to cruciferous plants. Host choice of these specialised insects is mediated by glucosinolates, which deter most other species but constitute important feeding and oviposition cues to Pieridae (Verschaffelt 1910, Fraenkel 1959b, Chew F.S. 1995). The fate of a plant in a plant-insect relationship was for a long time thought to have to passively endure herbivore attack (Schoonhoven 1996). Experiments with spider mites feeding on lima beans, however, lead to the idea that attacked plants defend themselves by mobilizing natural enemies of the attacking herbivore via herbivory-induced volatile emission (Dicke and Sabelis 1988, Vet and Dicke 1992, Dicke 1994). Similar tritrophic relationships were later found in corn (Turlings et al. 1990) and tobacco (Karban et al. 2000, Kessler and Baldwin 2001).

Among the chemical senses, olfaction plays a key role in host location for many insects (for review see Visser 1986, Ramaswamy 1988, Bruce et al. 2005a). If the antennae, the main olfactory organ of insects, were excised from females of the tobacco hawk moth *Manduca sexta* (Lepidoptera: Sphingidae), they could no longer find host plants for oviposition (Yamamoto and Fraenkel 1960). In 1989, Judd and Borden demonstrated the amazing sensitivity of the olfactory system to host plant emitted compounds. They were able to attract individuals of the onion fly, *Delia antiqua* (Diptera: Anthomyiidae) over a distance of 100 metres towards the characteristic host volatile compound dipropyldisulfide (Judd and Borden 1989). Plant volatiles do not only serve for long distance location, recently it was shown that grapevine moths *Lobesia botrana* (Lepidoptera: Tortricidae) are able to distinguish fungus-infected from healthy grapes by detecting the infection-specific compound 3-methyl-1-butanol (Tasin et al. 2012). Choice of host by the use of plant volatiles has been intensely investigated e.g. in parasitoids (Turlings and Benrey 1998, Mumm and Dicke 2010, McCormick et al. 2012). However, the contribution of olfaction to a mother's choice among suitable hosts for egg laying is poorly understood in herbivorous insects (Willis and Arbas 1991, Mechaber et al. 2002).

Therefore, this thesis and its underlying doctoral work focuses on the host choice of the oligophagous herbivore *M. sexta*, presenting insights into oviposition preferences, chemical identity of olfactory cues, and detection and processing of these choice-mediating semiochemicals[1]. In the following paragraphs, I will briefly review what is presently known regarding oviposition in insects, covering evolutionary aspects and previous knowledge on host plant use of *M. sexta*. I will also introduce the insect olfactory system.

Insect oviposition – choices and consequences

The aim to find favourable and secure conditions to benefit the offspring is ubiquitously spread among the world's creatures. Driven by evolution to maximize individual fitness by contributing to the gene pool of the next generation (Darwin 1859, Spencer 1866), parents seek to provide protection from a manifold of risks like predation or starvation. While many species take care of their offspring after hatching, parents of most insect species are dead before their offspring emerges (Cole 1954). Thus, the choice of adequate oviposition sites and hosts for the comparatively immobile offspring becomes vitally important for female insects (Figure 1) to increase offspring survival, reproductive success and, consequently, maximize inclusive individual fitness.

[1] semiochemical (greek semion: sign, signal and chemeceia: alchemic), introduced 1971 by Law and Regnier (1971) for chemical compounds mediating interactions between organisms.

Various physiological and ecological factors like nutritional quality (Scriber and Slansky 1981), palatability (Cornell and Hawkins 2003), and shelter from enemies (Bernays and Graham 1988, Dicke 1994) affect the fitness of herbivores feeding upon host plants (Ehrlich and Raven 1964). To find the optimal environment for their offspring, ovipositing insects need to take these criteria into account (Courtney et al. 1989, Thompson and Pellmyr 1991a, Mayhew 1997). Their influence on host choice within the same plant species has been studied extensively in a multitude of insect species (Thompson and Pellmyr 1991a, Mayhew 1997, Dicke 2000, Awmack and Leather 2002, chapter I, II). However, nature is rich in species and often offers more than one suitable host to an ovipositing female. Hence, oligophagous and polyphagous insects looking for an oviposition host occasionally might end up spoilt for choice at the botanical buffet. To avoid getting torn between potential hosts, a female surrounded by host plants of different species needs to revert on inherent or acquired preference hierarchies (Mayhew 1997, Riffell et al. 2013). For *M. sexta* preference hierarchies have been examined for part of its host plant spectrum, both between species (Yamamoto and Fraenkel 1960, Sparks and Cheatham 1970, Yamamoto 1972) as well as for different plant qualities within species (Kessler and Baldwin 2001, Reisenman et al. 2010). However, to date it remains unclear, whether choice between hosts on different preference hierarchy levels are mediated by olfactory or visual (from a distance) or gustatory or tactile cues (during contact). The question arises, how this preferences are

Figure 1. Predation risk of *Manduca* offspring
Eggs and larvae of *Manduca* moths are exposed to various sources of predation, and their survival depends on their mother's host choice. In the Utah desert *Geocoris pallens* (left, Hemiptera: Lygaeidae) constitutes the main predator of *Manduca* offspring. The hemipteran bug is attracted to herbivory-induced plant volatiles emitted upon larval damage (Allmann and Baldwin 2010). *Manduca* females detect the volatile signal as well and avoid these plants for oviposition (Kessler and Baldwin 2004, chapter II).
Photos: left, Merit Motion Pictures, Winnipeg, 598 Manitoba, Canada; right, D. Kessler

established at the sensory, processing and behavioural level. Which cues communicate different levels of attractiveness towards an ovipositing insect?

Manduca sexta and its host plants

Since half a century the tobacco hawk moth *M. sexta* serves as model system for insect olfaction (Yamamoto and Fraenkel 1960, Hanson and Dethier 1973, Matsumoto and Hildebrand 1981, Shields and Hildebrand 2000, Reisenman et al. 2004a), and host and food search (Yamamoto and Fraenkel 1960, Mechaber et al. 2002, Riffell et al. 2009, Reisenman et al. 2010). Oviposition of *M. sexta* is characterized by several sequential steps influenced by multiple sensory cues (Yamamoto et al. 1969). Visual stimuli were mainly investigated in association with nectar feeding (Goyret et al. 2007, Goyret et al. 2008), which is often reported to precede oviposition (Adler and Bronstein 2004). Behavioural assays using artificial plants demonstrated that chemical compounds extracted from the host plant trigger oviposition (Sparks and Cheatham 1970) and are detected by the insect antenna (Tichenor and Seigler 1980). Furthermore, wind tunnel experiments indicated an effect of olfactory cues on host plant location and approach (Mechaber et al. 2002, Fraser et al. 2003). However, the studies so far were dominated by no-choice experiments. Hence, the relevance of olfaction for host preference and host choice is still to be clarified (chapter I, III).

M. sexta occurs in arid areas of North and South America. Adult tobacco hawk moths use a wide range of plant species (Solanaceae, Agavaceae, Asteraceae, Fabaceae, Nyctaginaceae, Alarcón et al. 2008a) for nectar feeding and represent a major pollinator for several solanaceous plant species including *Datura wrightii* (Alarcón et al. 2008a). However, oviposition occurs almost exclusively on solanaceous plants (Mechaber and Hildebrand 2000, Mira and Bernays 2002). Three distinctive species are especially important: jimsonweed *D. wrightii* (Solanaceae, Yamamoto and Fraenkel 1960), devil's claw *Proboscidea parviflora* (Martyniaceae, Mechaber and Hildebrand 2000, Mira and Bernays 2002) and coyote tobacco *Nicotiana attenuata* (Solanaceae, Yamamoto and Fraenkel 1960).

In the Great Basin of Utah, *N. attenuata* and *D. wrightii* grow side by side constituting a major oviposition resource (Figure 2, Kessler 2012). Both are involved in an ambiguous relationship with *M. sexta* and the congeneric *M. quinquemaculata* by attracting the moths as pollinators (Alarcón et al. 2008a) but defending themselves against herbivory by *Manduca* larvae. *N. attenuata* has evolved highly specific adaptations to its most detrimental herbivore (Baldwin et al. 2001, Kessler and Baldwin 2001, Kessler and Baldwin 2004). Upon feeding damage these plants produce and emit herbivore-specific feeding-damage-induced volatile blends that are

used as kairomones[2] by predators (Halitschke et al. 2001, Kessler and Baldwin 2001, Allmann and Baldwin 2010). This emission of induced volatiles acts in concert with direct defences via secondary metabolites, leading to reduced larval performance and survival (Kessler and Baldwin 2004). *Manduca* moths also respond to the induced volatile emission by decreased oviposition on feeding-damaged plants as was shown in field (Baldwin et al. 2001, Kessler and Baldwin 2001) as well as in laboratory studies (chapter I, Reisenman et al. 2009).

Figure 2. Host plants of *M. sexta* in the Utah desert
N. attenuata (left) and *D. wrightii* (right) constitute two host plants for both, nectar feeding and oviposition (Kessler 2012, chapter I). They can differ considerably in size. Photos: left, C. Diezel; right, D. Kessler.

For *D. wrightii*, the investigation of factors affecting oviposition so far focused on flowers and floral volatiles. While there seems to be a positive relationship between the presence of flowers and oviposition (Adler and Bronstein 2004), the (+)- and (-)-enantiomers of linalool have antagonistic effects on egg deposition on flowering *D. wrightii* (Reisenman et al. 2010). But how does herbivory influence host choice among *D. wrightii* plants, which are especially attractive to *Manduca* moths (chapter I, III, Madden and Chamberlin 1945)? In contrast to *N. attenuata*, feeding-induced blends of volatiles from *D. wrightii* were reported to not consistently differ between herbivore species and are rather a general indicator of herbivory (Hare and Sun 2011a). However, in *N. attenuata* the emission of a herbivore-specific volatile signal coded by changes in proportions of two configurations of green leaf volatiles (GLVs) was caused by *M. sexta* larvae themselves (Allmann and Baldwin 2010) and thus, could be present also in other host plants. We

[2] Kairomone (greek kairos: opportunistic, exploitative), termed by Brown et al (1970) for interspecifically acting semiochemicals, which favour the receiver of the odorous message.

examined whether differences in plant quality modify female oviposition choice (chapter I, II) in both *N. attenuata* and *D. wrightii*, and how olfactory cues are involved in this behaviour. Volatile emissions of both host plants were investigated in response to larval feeding-damage, and oviposition experiments were performed in laboratory and field (chapter I, II). Host choice in response to herbivory was found to be host species dependent (chapter I). In chapter II we detected herbivory-specific changes in GLV emission affecting oviposition on *D. wrightii*.

Olfaction in Insects

Insects detect volatile compounds using olfactory sensory neurons (OSNs) located mainly on the antennae (Figure 3A). The OSNs are housed in sensilla, porous cuticular structures on the antenna. In moths single walled slender trichoid and cone-shaped basiconic outnumber double-walled pitted coeloconic sensilla. Other sensillum types may occur or even dominate in other insect taxa. However, the multiporous cuticle is a common feature to all olfactory sensilla. In males trichoid sensilla are predominantly dedicated to pheromone detection, whereas female trichoid sensilla are often associated with host plant odours (Shields and Hildebrand 2000).

One sensillum mostly contains one-to-three OSNs, each typically expressing one olfactory receptor (OR) type. Insect ORs possess a unique configuration as they are composed of a specific ligand-binding receptor (OrX) and the ubiquitous co-receptor (OrCo, Larsson et al. 2004, Vosshall and Hansson 2011), which is highly conserved in insect species (Jones et al. 2005). Both receptors form a heterodimer, which functions as ligand-gated ion channel (Sato et al. 2008, Wicher et al. 2008) translating incoming odour information into transducable action potentials. Upon odorant binding OrX activates OrCo via two pathways: an ionotropic pathway, which rapidly detects high odour concentrations and a metabotropic pathway that dependent on G proteins allows highly sensitive odour detection (Wicher et al. 2008, Hansson et al. 2010, Getahun et al. 2013). The metabotropic component and especially the role of G proteins during signal transduction is still controversially discussed (Nakagawa and Vosshall 2009, Wicher 2010, Martin and Alcorta 2011). OSNs send their axons to the antennal lobe (AL), the first olfactory neuropil of the insect brain. The AL consists of spherical centres of high synaptic density, so-called glomeruli. OSNs expressing the same OR converge onto the same glomerulus (Gao et al. 2000, Vosshall et al. 2000). Optical imaging takes advantage of this stringent OSN-glomerulus targeting and visualizes odour detection and AL processing as spatial patterns of neuronal activity (Joerges et al. 1997, Galizia et al. 1999, Sachse and Galizia 2002a, Hansson et al. 2003a, Skiri et al. 2004a, Carlsson et al. 2005, Silbering and Galizia 2007a). The organization of the AL

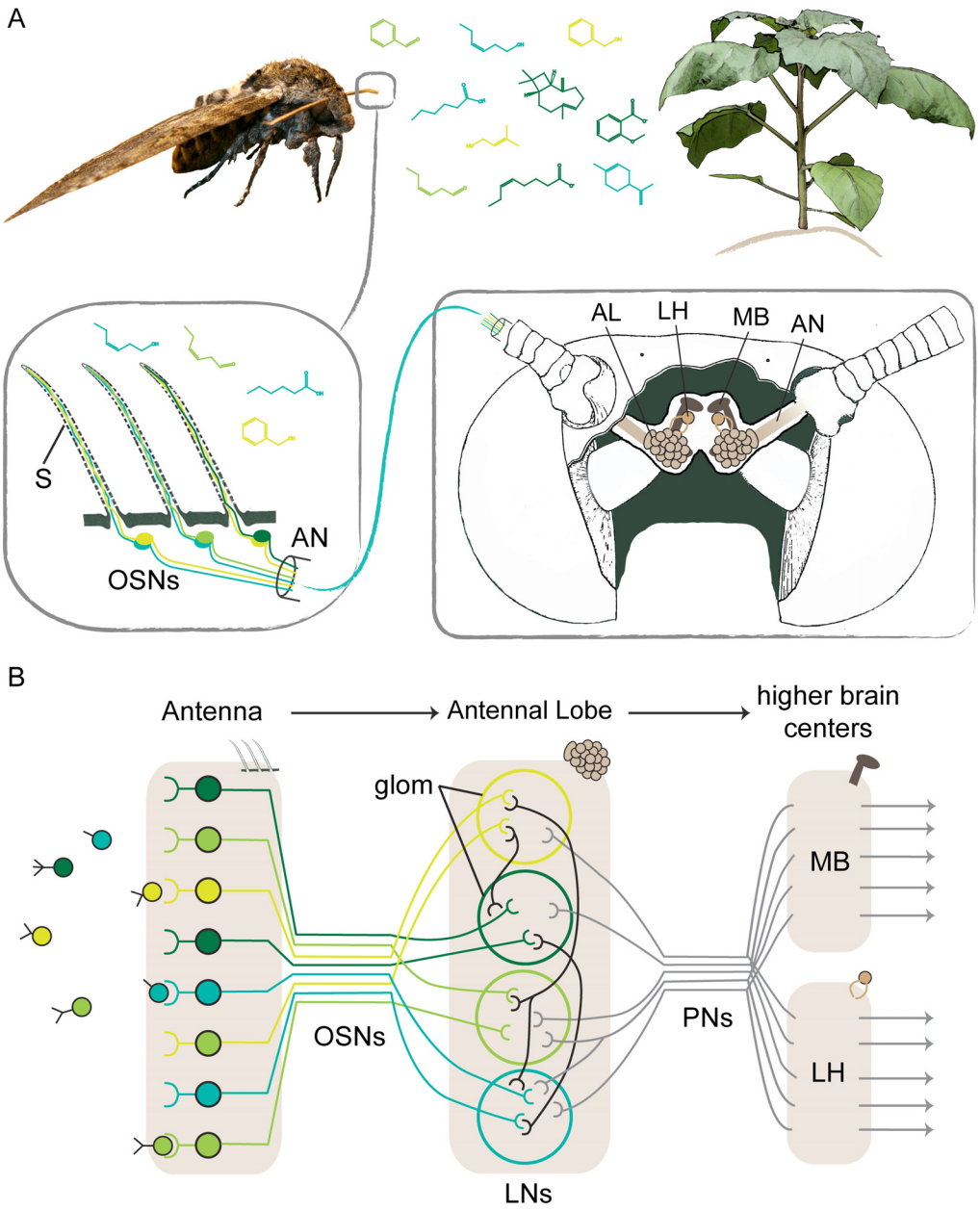

Figure 3. Odour perception and processing in the insect olfactory system
A. Odour molecules enter the olfactory system via thin wall pores in sensilla (S), where they bind to specific olfactory receptors located in the membrane of dendrites of olfactory sensory neurons (OSNs). Upon ligand binding OSNs generate action potentials that are conveyed to the first olfactory processing centre, the antennal lobe (AL), via axons in the antennal nerve (AN). Modified from Hansson (1999). B. OSNs expressing the same receptor target the same glomerulus (glom) leading to spatially distinct odour representation of neuronal activity in the AL. Lateral interneurons (LNs) interconnect glomeruli, establishing first odour processing. Projection neurons (PNs) relay odour information to higher brain centres (mushroom bodies (MB) and lateral horn (LH)). Modified from Vosshall and Stocker (2007).

is combinatorial: via OSNs individual odorants can activate a specific set of glomeruli, while each glomerulus can be activated by one to several odorants depending on the tuning of the OR expressed in the OSN. This combinational strategy allows the representation of an enormous variety of odorants by few coding units (Sachse and Krieger 2011).

First information processing (Figure 3B) takes place in the AL, established by a circuitry of lateral interneurons (LNs) which are restricted to the AL. LNs interconnect the glomeruli and alter the incoming information, mostly by inhibition (Homberg et al. 1989, Hildebrand and Shepherd 1997, Hansson and Anton 2000, de Bruyne and Baker 2008). Projection neurons (PNs) relay this information to higher brain centres like the mushroom bodies (MB) or the lateral horn (LH).

An insect searching for food or oviposition sites encounters a cacophony of scents. This necessitates an olfactory system that effectively detects, filters, and evaluates olfactory information to generate adequate odour-mediated behaviour. Differentially tuned OSNs establish an efficient filter at the periphery to assure selectivity of the olfactory system. How are then host species represented by the olfactory system? The expression olfactory 'gestalt' (Dethier 1974) refers to the idea, that a host blend is not perceived as single compounds but by a total pattern resulting in an odour image of the host species in the insect's brain. Which role do specific compounds or compositions play in host recognition and discrimination of *M. sexta*? In chapter I we show that differently tuned OSNs allow *M. sexta* females to distinguish host species and physiological conditions via the respective volatile profile. Furthermore, Ca^{2+} imaging of the female AL reveal that for an herbivory-induced compound class OSNs are configuration-specific (chapter II). We identified configuration-specific glomeruli in the female AL responding to two configurations of the same compound, respectively. In chapter III we use wind tunnel experiments to demonstrate the importance of host blend composition for host recognition.

Methodology

To understand how host plant volatiles influence host choice we used distinct physiological approaches addressing the peripheral and the central sensory system of *M. sexta*. At the periphery we challenged OSNs housed in individual sensilla with natural host blends at biologically relevant concentrations using gas chromatography coupled single sensillum recordings (GC-SSR, chapter I, Figure 4 blue box). In the antennal lobe we examined glomerular activation in response to single behaviourally relevant odours by optical imaging of OSN input (chapter II, Figure 4 pink box).

In contrast to electroantennographic (EAG) recordings (Schneider 1957), which pick up physiological responses of the antenna as a sum of all membrane potentials between recording and reference electrodes, single sensillum recordings (SSR, Schneider and Boeckh 1962) allow to determine the responsive range of individual sensilla or at the best of each OSN present in a sensillum. Linked to gas chromatography (Roelofs et al. 1971, Arn et al. 1975), these methods allow the identification of physiologically active volatiles and constitute a standard tool in

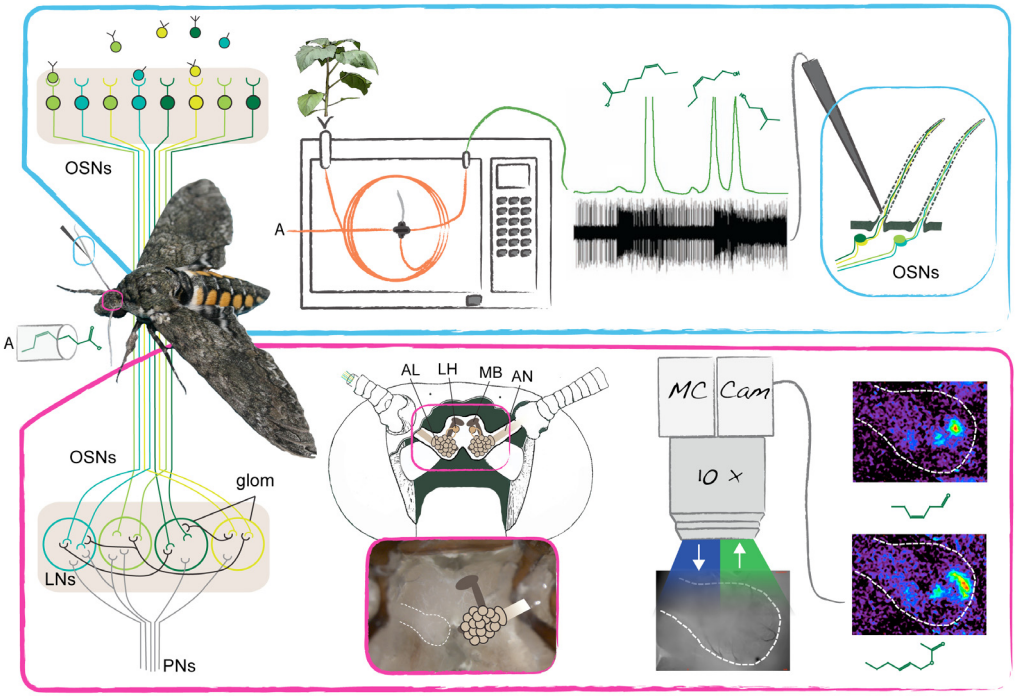

Figure 4. Physiological techniques investigating the *M. sexta* peripheral and central sensory system.
During GC-SSR experiments (blue box) the insect antenna is stimulated with a host volatile blend separated into single odours by a GC column. Tungsten electrodes inserted at the base of the sensilla measure the voltage fluctuation in the sensillum lymph, and pick up action potentials generated by the sensory neuron (spike trace). In optical imaging experiments (pink box) the increase of cellular Ca^{2+} is measured via fluorescence microscopy using a Ca^{2+} sensitive dye that is bath applied to the brain of living insects. Ca^{2+} signals, representing neuronal activation, are visualized in false-colour coded images.

Chemical Ecology to investigate the receptive range of insects (Fraser et al. 2003, Bengtsson et al. 2009). While EAG is a rapid and convenient screening technique, SSR has an inherently improved signal to noise ratio (Wibe 2004). Screening many individual sensilla with biologically relevant odour sets reveals the tuning width of single neurons and allows classification of OSN types based on their response profiles (Shields and Hildebrand 2000, chapter I), and conclusions concerning the transmission of olfactory information to the AL (chapter I).

While the SSR technique displays odour responses of individual OSNs, functional imaging of the AL reveals the global pattern of OSN activation. Neural activity in response to odour stimulation is detected via a Ca^{2+} sensitive dye, which changes fluorescence upon binding of Ca^{2+} ions (Galizia et al. 1997). Due to stringent OSN-glomerulus targeting, odour stimulation results in chemotopic maps of spatiotemporal neuronal activity in the AL (Galizia and Menzel 2001). The emerging activity pattern of all (visible) OSN populations facilitate comparisons of OSN activation on a global level and may reveal plasticity in the perception of host volatiles (Barrozo et al. 2011, Saveer et al. 2012) as well as blend interactions (Pinero et al. 2008, Kuebler et al. 2012). By comparing neural activity patterns we identified two configuration-specific OSN populations selectively responding to the (*Z*)-3- or (*E*)-2-configuration of the same compound.

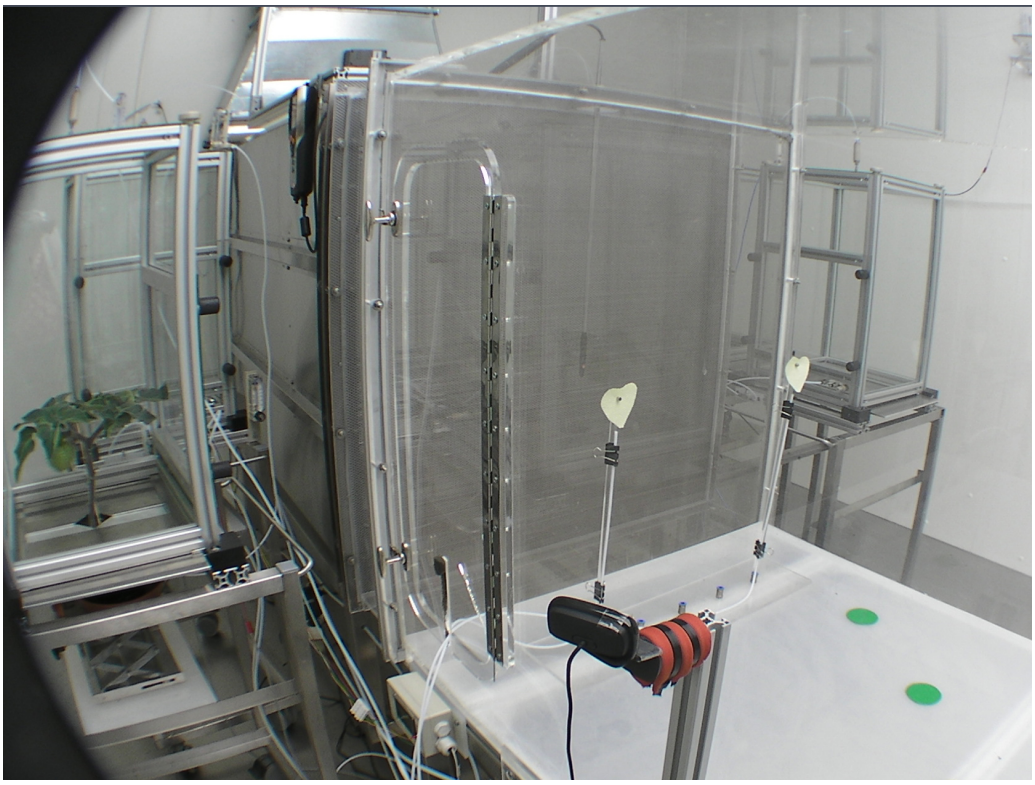

Figure 5. Wind tunnel setting for the investigation of host choice of *M. sexta* females.
The choice of mated *M. sexta* females between two odour sources presenting host plant headspace, clean air or manipulated plant headspace was investigated in wind tunnel experiments. All except olfactory host cues were excluded. On-line produced host plant headspace was transferred from a glass box into the wind tunnel and released at surrogate leaves. Photo: M. Knaden

Wind tunnel experiments with insects have been performed for more than 50 years and enabled the identification of behaviourally active chemicals acting as attractant or repellent to flying insects (Kellogg and Wright 1962). The technique facilitated the identification of sex pheromones for several insect species (Tumlinson et al. 1989, Baker et al. 1991, Doolittle et al. 1991, Acin et al. 2010) and highlighted the behavioural role of pheromone blends (Baker and Linn 1984). The attraction of female insects to host plant volatiles has been studied in wind tunnels as well (Mechaber et al. 2002, Fraser et al. 2003, Proffit et al. 2011, von Arx et al. 2011). However, stimulus delivery did often not reflect natural conditions, or other than olfactory stimuli were available to the insect as well. Host volatiles have been represented by plants (Kalberer et al. 2010, Proffit et al. 2011) or plant parts (Willis et al. 1995, Saveer et al. 2012) placed at the upwind end of the tunnel, by artificial mixes of host compounds (Cha et al. 2008, von Arx et al. 2011) or headspace extracts presented on a filter paper (Fraser et al. 2003). In our experiments we presented host odours via on-time produced plant headspace, which was transferred from plant housing glass boxes to surrogate leaves in the tunnel (chapter III, Figure 5). Consequently, we excluded all host-related stimuli apart from olfactory cues.

Volatile mediated interactions

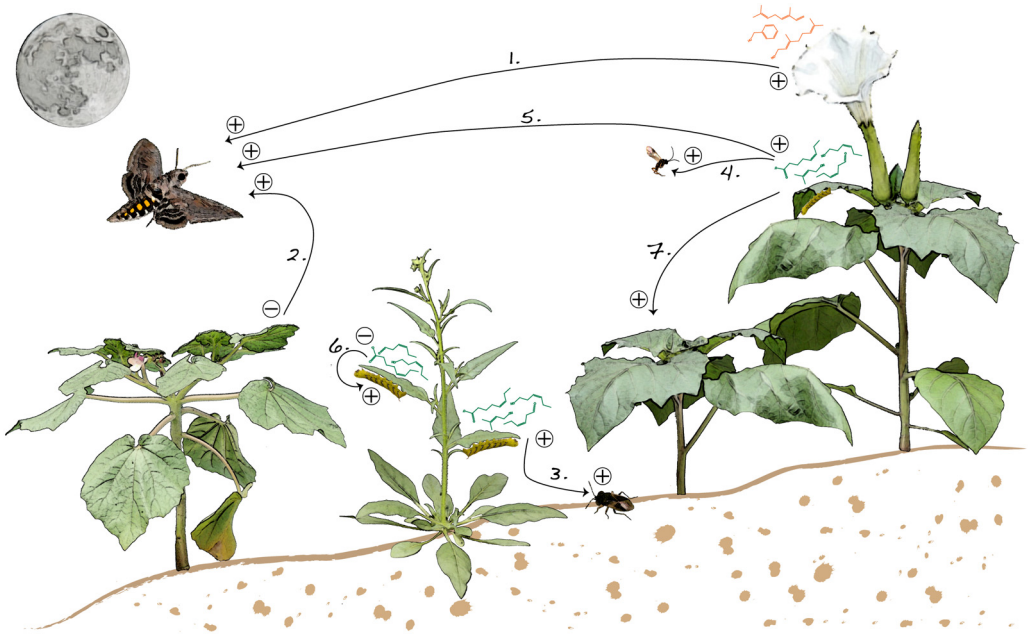

Figure 6. Semiochemically mediated interactions between *Manduca sexta* and its host plants
Host plant odours attract *M. sexta* moths and mediate host choice (chapter I, III). They can be beneficial (+) or adverse (-) to their emitter and receiver. Floral odours are beneficial (+) for moths and plants. They attract moths (1), which feed on nectar and serve as pollinator to the plant (Riffell et al. 2013). *M. sexta* benefits from vegetative (2) and floral (1) host volatiles for oviposition resulting in plant damage (2) due to larval herbivory. Herbivore-induced plant volatiles (HIPVs) emitted upon feeding damage by *M. sexta* larvae act as indirect plant defence. They attract predators (3) and parasitoids (4) of the larvae (Mira and Bernays 2002, Allmann and Baldwin 2010). Ovipositing *M. sexta* females (5) take advantage of HIPVs as well and avoid HIPV-emitting plants (2, Kessler and Baldwin 2004, chapter I). In contrast, HIPVs function as phagostimulants for the feeding larva (6, Halitschke et al. 2004). Neighbouring plants benefit by priming their defence systems (7) in response to HIPV detection (Heil and Karban 2010) .

M. sexta females navigate through a chemical world. Semiochemicals emitted from host- and non-host plants, influenced by plant-feeding conspecifics or other insects, deliver odorant messages to the moth. Figure 6 illustrates chemically mediated interactions showing how one signal can have several effects depending on the receiver. Flower volatiles attract foraging moths, which serve as pollinators for the plant (Bronstein et al. 2009, Riffell et al. 2009). We show that vegetative host plant odours attract ovipositing females and mediate host choice (chapter I, III). Herbivore-induced plant volatiles (HIPVs), released upon feeding damage by *M. sexta* larvae (Kessler and Baldwin 2004, Hare and Sun 2011a), play several roles depending on the receiver (Dicke and Baldwin 2009). To the plant-consuming larva, HIPVs function as feeding stimulant (Halitschke et al. 2004). Neighbouring plants take advantage of volatile emissions by priming their defence upon HIPV perception (Heil and Karban 2010). But most important for this study, by attracting predators and parasitoids of the larvae, HIPVs act as indirect plant defence against herbivores (Kessler and Baldwin 2001). Ovipositing *M. sexta* females recognize the

volatile 'cry for help' (Dicke and Baldwin 2009) and have been shown to avoid HIPV-emitting plants (chapter I, Kessler and Baldwin 2004).

In chapter I we examine the host preference of mated *M. sexta* females to three ecologically relevant host species. We show that the observed preference hierarchy is mediated by vegetative plant volatiles (chapter I, III). We also show that ovipositing *M. sexta* females prefer intact over feeding damaged plants. This feeding damage dependent choice, however, is dependent on host species (chapter I). We demonstrate, that the moth's peripheral sensory systems is endowed to differentiate between volatile blends of intact and feeding damaged plants (chapter I) and that the system furthermore is able to distinguish different origins of plant damage by detecting a shift between the configurations of one emitted plant compound (chapter II). Wind tunnel experiments confirm the prominent role of olfaction during host choice (chapter III) showing that host blends alone are sufficient for *M. sexta* females to choose their preferred host. Ovipositing moths seem to perform host choice using a host-specific olfactory 'gestalt' (chapter I, III), which is mainly dependent on blend composition but can be influenced by volatile signal intensity as well. This thesis highlights how host plant derived volatile semiochemicals mediate host preference and host choice by ovipositing *M. sexta* females via a highly sophisticated olfactory system.

OVERVIEW OF MANUSCRIPTS

Chapter I

Plant Species- and Status-specific Odorant Blends Guide Oviposition Choice in the Moth *Manduca sexta*

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[*]*These authors contributed equally to the work.*

Chemical Senses, accepted September 19, 2012

Zur Publikation angenommen

In the first chapter, we show that the host preference of ovipositing *Manduca sexta* females is influenced by host species as well as host condition and that differences in species and damage status are reflected in the plant volatile emission. Peripheral sensory neurons screened for their response to these species- and status-specific volatile blends allow separation of host species as well as damage status by their response profile. Thus, we present the capability of an insect olfactory system to distinguish host plants of different quality and suitability to nourish and shelter the insect's offspring thereby ensuring reproductive success.

Built on an idea conceived by all authors.
designed experiments: A. Späthe (30%), A. Reinecke, S. Olsson, M. Knaden, B. Hansson
conducted behavioural experiments: A. Späthe (80%), A. Reinecke
performed and analysed plant volatile collections: A. Späthe (100%)
performed and analysed SSR-experiments: S. Kesavan , A. Reinecke, S. Olsson
wrote the manuscript: A. Späthe (20%), A. Reinecke, S. Olsson, M. Knaden, B. Hansson

Chapter II

Feeding-induced rearrangement of green leaf volatiles reduces moth oviposition

Silke Allmann[*], Anna Späthe[*], Sonja Bisch-Knaden, Mario Kallenbach, Andreas Reinecke, Silke Sachse, Ian T. Baldwin and Bill S. Hansson

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eLIFE, accepted April 12, 2013

Zur Publikation angenommen

The second chapter showed that *M. sexta* can distinguish between mechanically and feeding-damaged *D. wrightii* plants. The latter emit a herbivory-specific signal characterized by increased emission of (*E*)-2-configurated green leaf volatiles resulting in a low (*Z*)-3/(*E*)-2-ratio of these compounds, which has been shown to attract predators of *Manduca* offspring. In optical imaging experiments with *M. sexta* females we found configuration-specific OSN populations responding to either (*Z*)-3- or (*E*)-2-hexenyl acetate. In field experiments *Manduca* moths oviposited less on plant sites that emitted a low (*Z*)-3/(*E*)-2-ratio of green leaf volatiles most probably to avoid larval feeding competition and increased predation risk.

Built on an idea conceived by all authors.
designed experiments: S. Allmann, A. Späthe (30 %), S. Bisch-Knaden, M. Kallenbach
performed field behavioural experiments: S. Allmann and I. Baldwin
performed imaging experiments: A. Späthe (70%) and S. Bisch-Knaden
performed imaging data analysis. A. Späthe (70%) and S. Bisch-Knaden
collected field volatile data: S. Allmann and M. Kallenbach
collected laboratory volatile data: S. Allmann and A. Späthe (50%)
performed analysis of all volatile analysis: S. Allmann and A. Späthe (50%)
wrote the manuscript: S. Allmann, A. Späthe (40%), I. Baldwin and B. Hansson

Chapter III

Signal intensity affects host selection in *Manduca sexta*

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[*]*These authors contributed equally to the work.*

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The third chapter reports on wind tunnel experiments presenting exclusively plant headspace volatiles of two differently preferred host plants to mated *M. sexta* females. The observed host preferences were consistent with previously reported oviposition preferences. Furthermore, manipulation of the volatile intensity of the less preferred host plant revealed an effect of volatile signal intensity on host preference. Thus, *M. sexta* females perform their host choice using a host-specific olfactory 'gestalt', which is mainly dependent on blend composition but can be influenced by volatile signal intensity as well.

Built on an idea conceived by all authors.
designed experiments: A. Späthe (30%), A. Reinecke, M. Knaden
performed wind tunnel experiments: A. Späthe (60%), A. Reinecke and A. Haverkamp
performed data analysis: A. Späthe
wrote the manuscript: A. Späthe (60%), A. Reinecke, M. Knaden and B. Hansson

CHAPTER I

Plant Species- and Status-specific Odorant Blends Guide Oviposition Choice in the Moth *Manduca sexta*

Anna Späthe*, Andreas Reinecke*, Shannon B. Olsson, Subaharan Kesavan, Markus Knaden and Bill S. Hansson

**These authors contributed equally to the work.*

M. sexta larva and eggs on *Nicotiana attenuata*. Photo: A. Späthe

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Plant Species- and Status-specific Odorant Blends Guide Oviposition Choice in the Moth *Manduca sexta*

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Abstract

The reproductive success of herbivorous insects largely depends on the mother's oviposition preference. In nocturnal insects, olfaction is arguably the most important sensory modality mediating mate finding, foraging, and host location. In most habitats, gravid females select among a number of plants of varying suitability, yet assessment of the neuroethological mechanisms underlying odor-guided choice between host plants is rare. Using a series of behavioral, electrophysiological, and chromatographic analyses in the Hawk moth, *Manduca sexta*, we show that gravid females perform a hierarchical choice among host plants of different species and qualities using olfactory cues. Both relevant plant species and qualities can be distinguished by volatile profiles collected from the headspace of these plants, and olfactory sensilla on female antennae detect more than half of the about 120 analytically detected volatiles in host plant headspace samples. Although olfactory sensory neurons present in antennal sensilla are mainly broadly tuned to multiple host compounds, some sensilla exhibit species and condition-specific responses. In fact, species and quality can be distinguished by the physiologically active components alone. Our findings thus suggest that distinguishing characteristics of both host species and quality are already represented at the sensory periphery.

Key words: *GC-SSR*, host plant choice, *Manduca sexta*, moth, olfaction

Introduction

For animals that do not directly supply food and care to the next generation, the choice of brood site becomes vital for reproductive success. Early life stages are often less mobile (e.g., eggs or larvae), and female substrate choice becomes instrumental in placing eggs in a nourishing and protective environment. Most insects fall into this category, in which an egg-depositing female is often dead when the offspring emerge. For insects accepting several plant species, different criteria define a suitable larval host, for example, palatability, nutritional quality, and shelter from enemies (Courtney et al. 1989; Thompson and Pellmyr 1991; Mayhew 1997). Additionally, ovipositing females encounter ongoing herbivory and predation risks augmented by the plant's induced defense system further shaping host preference (Murphy 2004; Singer et al. 2004). As most moths are nocturnal and/or crepuscular, they have developed a high dependence on olfactory input. This consequently necessitates an olfactory system designed to detect odor cues indicating host plant identity and quality. A number of studies (Hansson et al. 1999; Kalinova et al. 2001; Shields and Hildebrand 2001; Røstelien et al. 2005) have characterized the specificity and sensitivity of antennal olfactory sensory neurons (OSNs) in detecting plant-related odor information and delivering it to early processing centers of the brain. Responses to individual host species or specific volatile compounds have also been investigated (Matsumoto and Hildebrand 1981; Eisthen 2002; Mechaber et al. 2002; Fraser et al. 2003; Reisenman et al. 2004). Direct comparisons of choice among host plants and olfactory reception are, however, rare, and even more so when considering ecologically relevant interactions.

Manduca sexta is a large sphingid moth primarily occurring in arid areas of North and South America. It has been studied as a model system both for insect olfaction (Matsumoto and Hildebrand 1981; Shields and Hildebrand 2001; Eisthen 2002; Reisenman et al. 2004) and for host and food search (Yamamoto and Fraenkel 1960; Mechaber et al. 2002; Fraser et al. 2003; Riffell et al. 2009; Reisenman et al. 2010). These moths choose within a range of host plants, with 3 highly distinctive species playing important roles: *Datura wrightii* (jimsonweed) (Yamamoto and Fraenkel 1960), *Proboscidea parviflora* (devil's claw) (Mechaber and Hildebrand 2000; Mira and Bernays 2002), and *Nicotiana attenuata* (wild tobacco) (Yamamoto and Fraenkel 1960). In the Great Basin of southwestern Utah, United States of America, the impact of *Manduca* larvae in the local ecosystem has been studied in detail (Baldwin et al. 2001). Classic experiments with *N. attenuata* have shown how manipulations of plant volatile emissions can have profound effects on recruitment of the herbivore and its predators (Kessler and Baldwin 2001). Congeneric *M. quinquemaculata* prefer to oviposit on undamaged *N. attenuata* plants compared with plants damaged by feeding *M. sexta* larvae (Baldwin et al. 2001; Kessler and Baldwin 2001). Damaged *N. attenuata* plants in turn produce blends of induced volatiles that are specific for the feeding herbivore, and are used as signals by predators (Halitschke et al. 2001; Kessler and Baldwin 2001). This emission of induced volatiles in concert with direct defenses via secondary metabolites also leads to decreasing larval performance and survival (Kessler and Baldwin 2004). Feeding-induced blends of volatiles from *D. wrightii*, in contrast, do not consistently differ between herbivore species and are rather a general indicator of herbivory (Hare and Sun 2011).

To assess the role of plant odor cues in these condition- and species-dependent oviposition choices, we performed behavioral, chemical, and linked chemical-electrophysiological investigations of the herbivore *M. sexta*. The main questions were as follows: 1) Do females exhibit a choice for oviposition when provided with a selection of host plant species and qualities? 2) Is this choice based on olfactory input? 3) Which plant chemicals potentially constitute the semiochemicals emitted by the plant? and 4) Which of these are detected by OSNs on the female moth antenna? Together our results show that host plant choice exhibited by female *M. sexta* moths is reflected in species- and quality-specific volatile emissions detected by a combinatorial population of both selective and nonselective antennal OSNs.

Materials and methods

Insect rearing

Adult *M. sexta* were maintained at ambient conditions and provided with *N. attenuata* plants for oviposition. Eggs and larvae were kept at 27 °C and 70% humidity on artificial diet (Grosse-Wilde et al. 2011). Wandering fifth instar larvae were separated individually and pupae kept within climate chambers until sexing 1 week before emergence. Individual females were mated during the third night after emergence and used in behavioral experiments the fourth or fifth night. Mating success was confirmed by allowing deposited eggs to develop into vital larvae. All rearing facilities for larvae, pupae, and adults were devoid of any plant material. Adults had unrestricted access to sugar solution in artificial flowers.

Plant breeding

All plants were grown in a greenhouse (23–25 °C, 50–70% relative humidity, 16 h light, Philips Sun-T Agro 400 W Na vapor bulbs, 350–500 µmol/m^2/s photosynthetic photon flux at plant level) until reaching a height of 30–40 cm and were used before flowering.

Datura wrightii seeds were purchased from B & T World Seeds and subsequently harvested from plants bred in the greenhouse. Plants were grown in 2-L pots and used in experiments 40–45 days after sowing.

Nicotiana attenuata seeds were obtained from an isogenic line that had undergone 30–31 generations of inbreeding and was originally derived from an accession collected from a burn in southwestern Utah. After germination (Krügel et al. 2002) seedlings were planted at day 10 in seed flats, transferred to 1-L pots at day 20, and grown under greenhouse conditions until use between 38–42 days after sowing.

Proboscidea parviflora seeds were initially collected from wild plants in southwestern Utah and subsequently harvested from plants grown in the greenhouse. *Brassica oleracea* var. *Rosella* seeds were purchased from Erfurter Samen und Pflanzenzucht GmbH.

Oviposition preference assays

About 1 h before onset of the scotophase, individual females were transferred into screened flight cages in a greenhouse (Figure 1). Females were tested individually. Sucrose solution was provided away from the experimental plants. Females moved freely and oviposited during 1 entire night. Individual egg numbers were assessed the following morning and compared applying a Friedman test and Dunn's Multiple Comparison test for multiple choice assays, and by a Wilcoxon signed-rank test for pairwise comparisons. All plants and females were used only once.

Choice of host species—oviposition on plants

Eight plants of the host species *D. wrightii, N. attenuata* (Solanaceae), and *P. parviflora* (Martinyaceae) and the non-host *B. oleracea* (Brussels sprout) were arranged in 2 patches per species for a total of 32 plants per flight cage. Patches were randomly distributed within the cages (Figure 1A). In total, 26 females were tested individually as described in

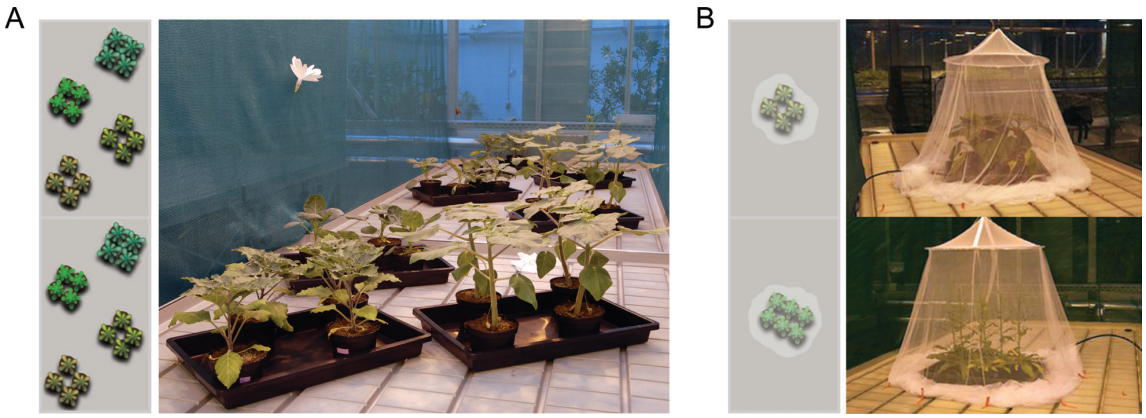

Figure 1 Oviposition preference assay. Individual *Manduca sexta* females were tested in a screened flight cage (7 × 1.8 × 2.3 m) in the greenhouse (23–25 °C, 50–70% relative humidity). Females were allowed to move freely and oviposit during the entire night. Deposited eggs were counted the following morning. (A) 8 plants of the host species *Datura wrightii, Nicotiana attenuata* (both Solanaceae), and *Proboscidea parviflora* (Martinyaceae), and the nonhost *Brassica oleracea* (Brussels sprout; Brassicaceae) were arranged in 2 patches per species for a total of 32 plants per flight cage. Patches were randomly distributed within the cages at distances of 1–1.5 m. (B) 4 *D. wrightii* and 6 *N. attenuata* plants, respectively, were placed under 2 circular gauze tents (top diameter 60 cm, base diameter 1.2 m) with an outlet of charcoal filtered air (30 L/min) at the center pushing volatile compounds out of the tent. Any contact between gauze and plants was avoided. This figure appears in color in the online version of *Chemical Senses*.

Table 1 Plant damage treatments

Treatment	Control	Plant signal	Larval signal
Larval feeding	Intact plants	Feeding damage–induced volatiles	Body and feces odors
Temporal larval damage	Intact plants	Feeding damage–induced volatiles	—
Mechanical wounding and spit	Intact plants	Volatiles induced by elicitor present in the regurgitant (Halitschke et al. 2001)	—
Mechanical wounding and chemical induction	Intact plants	Methyl jasmonate-induced volatiles (Halitschke et al. 2000)	—
Caged larvae and feces on intact plants	Intact plants and empty cages	Volatiles from intact plant	Body and feces odors

Oviposition preference assays above. Six females, which did not oviposit, were excluded from statistical analysis.

Choice of host species—contact excluded

Four *D. wrightii* and 6 *N. attenuata* plants, respectively, were placed under 2 circular gauze tents (Figure 1B) with an outlet of charcoal filtered air (30 L/min) at the center pushing volatile compounds out of the tent. Plant numbers were chosen to compensate for silhouette appearance. Any contact between gauze and plants was avoided. Twenty-seven gravid females were used. Sixteen females did not oviposit and were thus considered nonresponders.

Choice of host plant—effect of damage

The effect of feeding damage on female oviposition preference was investigated in *N. attenuata* and *D. wrightii*. Four patches with 3 feeding-damaged and 4 patches with 3 undamaged plants each (see Larval feeding) were placed into the flight cage as described in Oviposition on plants. A total of 29 and 27 individual females were allowed to choose between undamaged and larval feeding–damaged *N. attenuata* or *D. wrightii* plants, respectively. To increase robustness of within-plant species comparison, especially with respect to ratios, females ovipositing less than 10 eggs were excluded from statistical analysis in these experiments (*N. attenuata*, $N = 5$; *D. wrightii*, $N = 5$). Differential oviposition in response to feeding damage was only found for *N. attenuata*, see Results. Oral secretions (OS) may elicit herbivore species-specific volatile emissions in host plants (Halitschke et al. 2000). We therefore applied different treatments (see Table 1) to *N. attenuata* plants to furthermore elucidate whether volatiles nonspecifically emitted by the plant after mechanical damage and chemical defense induction, volatiles specifically emitted by the plant after larval feeding, volatiles emanating from the caterpillar or its feces, or volatiles from both plant and feeding caterpillars contribute to the behavioral preference for undamaged compared with feeding-damaged plants observed in *N. attenuata*. Experimental plants were treated as follows:

Larval feeding.

Three second- to third-stage *M. sexta* larvae were allowed to feed on *N. attenuata* and *D. wrightii* plants starting 48 h before and during the experiment. Control plants were left undamaged.

Temporal larval damage.

Manduca sexta larvae were allowed to feed on test plants starting 36 h before the experiments. Larvae were individually encaged at the undersides of leaves to prevent the accumulation of feces on the plant surface. Cages consisted of 40-mm outer diameter rubber foam rings containing small magnets and covered with wire mesh. The upper side of the leaf was covered with transparent plastic film of the same diameter fixed to the cage by a ferromagnetic steel ring. Control plants carried empty cages. Larvae and cages were removed before start of the experiment.

Mechanical wounding and spit.

Starting 36 h before the experiment, leaves were wounded once on each side of the mid-vein using a pattern wheel. OS from third to fourth instar *M. sexta* larvae (Halitschke et al. 2001) reared on *N. attenuata* plants (5 µL, 1:10 dilution in aqua dest) were pipetted onto the fresh punctures. The treatment was applied 3 times a day to a set of 3 leaves per day. Control plants were left undamaged.

Mechanical wounding and chemical induction.

Following the method described by Halitschke et al. (2000), 40 µL of Lanolin (Sigma Aldrich) containing 300 µg methyl jasmonate (Sigma Aldrich) were applied to the lower part of the elongating stem starting 36 h before the experiment. Leaves were mechanically damaged 4 times per day, see Mechanical wounding and spit above. Control plants were treated with pure lanolin paste and left undamaged.

Caged larvae and feces on intact plants.

Three third instar *M. sexta* larvae previously fed ad libitum on *N. attenuata* plants were placed into Nylon mesh cages (10 × 45-mm internal diameter [ID]; mesh size 1.2 mm; Exo Terra). Cages were fixed on wooden sticks next to undamaged plants 3–4 h before the experiment. Empty cages were placed next to control plants.

Volatile collection and analyses

Volatile collections were performed on whole plants for 1) semiquantitative analysis of the relative composition of odor bouquets emitted by different host plant species and conditions and 2) extracts at high concentrations to characterize the responsive range of OSNs when challenged with natural volatile stimuli. *Nicotiana attenuata* and *D. wrightii* plants were either intact or treated as stated in the Larval feeding section.

1) Headspace collections were performed in 25-L silanized glass cylinders. Teflon discs with a central opening (80-mm ID) separated plant shoots from soil and roots. Charcoal filtered air was introduced at the top (1.2 L/min). Odor-laden air was pulled (1 L/min) through outlets 5 cm above the Teflon disks connected to sorptive filters with 25 mg each of Carbotrap C, B, and X (Sigma Aldrich). Odor collections began 20 min after onset of the scotophase and ran for 6 h. The adsorbents were eluted with 1 mL dichloromethane (DCM) containing 1 µg bromodecane as internal standard (IS). Eluates were concentrated under a gentle stream of N_2 to 30 µL and stored at −80 °C. 2) For physiological studies, flow rates were adjusted to 2.2 (in) and 2 L/min (out), respectively, and volatiles were trapped during 12 h in the dark on 25 mg SuperQ (80/100 mesh, Supelco) or 25 mg activated charcoal as adsorbent. The adsorbent was eluted with 400 µL DCM, the eluate concentrated to 30 µL, and stored at −80 °C. Headspace extracts for gas chromatograph-coupled single sensillum recording (GC-SSR) contained compounds from the lower nanogram to the lower microgram range.

Analytical procedures

All volatile analyses were carried out on 7890A gas chromatographs (GC) (Agilent Technologies) operated in splitless mode, the injection port kept at 230 °C, and 1 µL of sample injected. For compound identification and semiquantification, both nonpolar and polar columns (HP-5 MS ui and Innowax; 30 m, 0.25-mm ID, 0.25-µm film thickness; J&W Scientific) operated under constant He flow (1.1 mL/min) were used with total ion chromatograms recorded by an Agilent 5975C mass spectrometer (MS). The GC oven was kept at 40 °C for 5 min, ramped at 5 °C/min to 260 °C or 280 °C for Innowax or HP-5 columns, respectively. The MS transfer line was maintained at 280 °C and the MS operated in electron impact mode (70 eV, ion source: 230 °C, quadrupole: 150 °C, mass scan range: 33–350 amu). Compounds were identified by comparing mass spectra and Kovats retention time indices to authentic reference compounds or tentatively to those published by the National Institute of Standards and Technologies. For semiquantification the GC-MS system was calibrated with the IS (33, 10, 5, 1, and 0.5 ng; $N = 3$ replicates). Emission rates were subsequently calculated based on comparison of peak areas of individual compounds and the IS and headspace sampling times. Volatile samples were compared by random forest analysis (Breiman 2001) performed in "R" (randomForest: Breiman and Cutler's random forests for classification and regression' package, version 4.5-34 for R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). Random forest analysis is similar to a principal component analysis but better suited for nonparametric data sets that include many more variables than samples (Breiman 2001). However, regardless of how well the 2 methods are suited for our analysis, random forest analysis and principal component analysis yielded comparable results (Figure 4 and Supplementary Figure 1). For each analysis, n_{tree} = 100 000 bootstrap samples were drawn with n_{try} set as sqrt(number of compounds). We

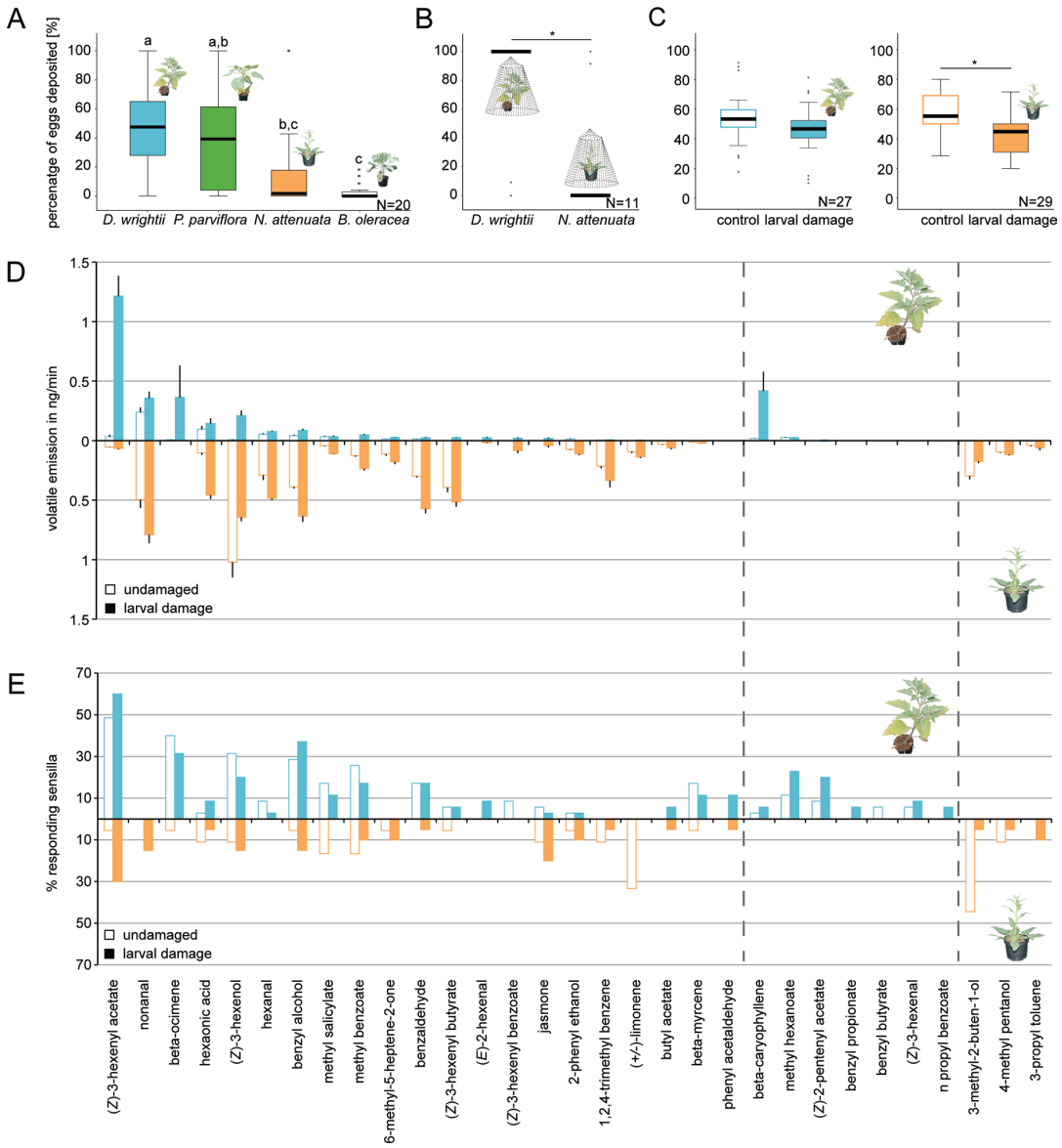

Figure 2 Oviposition preference and olfactory response of *Manduca sexta* females to host plants is species and condition specific. (A–C) Oviposition choice experiments. (A) In a flight cage, individual *M. sexta* females preferred to oviposit on nonflowering *Datura wrightii* and *Proboscidea parviflora* over *Nicotiana attenuata* and/or the nonhost *Brassica oleracea* (letters depict significant differences between species). (B) Individual females preferred to oviposit on gauze tents hiding *D. wrightii* compared with tents hiding *N. attenuata* plants. (C) Females preferred to oviposit on undamaged (open boxes) versus larval-damaged (filled boxes) *N. attenuata* plants (right), but did not prefer any *D. wrightii* plant state (left). (D) Average scotophase volatile emissions (ng/min) of *N. attenuata* (bottom) and *D. wrightii* (top) plants. Only compounds that elicited repeated responses in the GC-SSR experiments are listed. Dashed lines separate species-specific compounds. Error bars denote standard errors ($N = 5$). (E) Percentage of *M. sexta* sensilla responding in GC-SSR experiments using *N. attenuata* (bottom) and *D. wrightii* (top) headspace samples (Supplementary Table 2). Dashed lines separate species and condition-specific (filled vs. open bar) responses. This figure appears in color in the online version of *Chemical Senses*.

performed the analysis for all headspace volatiles and for those volatiles found to be active in the SSR experiments. In comparing SSR active with headspace volatiles (Figure 2), we only considered volatiles eliciting responses in at least 2 sensilla that were also detected in the semiquantitative headspace analyses, or those that elicited responses in at least 10% of recorded sensilla.

GC-SSR experiments were conducted on an instrument equipped with an HP-5 MS column (30 m, 0.32-mm ID, 0.25-µm film; J&W Scientific) at constant He flow (2 mL/min).

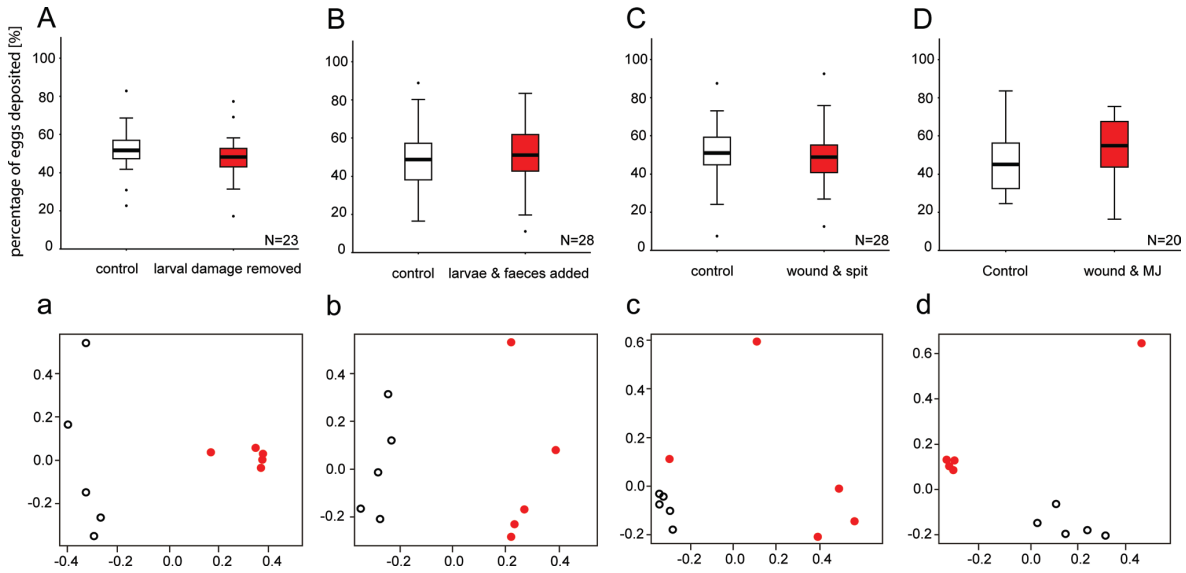

Figure 3 Oviposition preference requires complete volatile information on larval damage. (A–D) The oviposition preference of individual *Manduca sexta* females disappeared when offered different plant treatments (filled boxes) aiming to disentangle the volatile information emitted by the herbivore host complex. Experiments presented either (A) feeding-induced plant volatiles without larvae and feces, (B) larval and fecal odors only, (C) plant volatiles released after mechanical damage and application of *M. sexta* OS, or (D) plant volatiles released after mechanical damage and application of methyl jasmonate. (a–d) Metric multidimensional scaling representation of volatile blends from treated plants (filled circles) versus control plants (open circles) based on SSR-active volatiles (see Table 1). Random forest analysis revealed clear separation of treatment and control samples based on physiologically active compounds. This figure appears in color in the online version of *Chemical Senses*.

The oven was programmed at 40 °C for 1 min with a ramp of 20 °C/min to 280 °C held 10 min. The effluent flow was split 40:60 with make-up gas added (He, 30 psi) via a Gerstel 3D/2 connector (Gerstel) and inert restriction columns to synchronously reach a flame ionization detector (350 °C) and antennal preparations via a modified olfactory detection port (ODP3, Gerstel). The transfer line was kept at 280 °C.

Electrophysiological recordings—GC-SSR

Manduca sexta females (3–4 days after eclosion) were fixed dorsally with wax to glass slides in disposable 20 mL Falcon tubes with the head protruding. Electrode positioning was achieved using piezoelectric micromanipulators (PM-10 Märzhäuser). Electrolytically sharpened tungsten electrodes connected to a 10× preamplifier head stage (Syntech) were inserted randomly into the base of trichoid and basiconic olfactory sensilla with a reference electrode placed in the eye. Signals from OSNs were digitized by an IDAC-4 interface (Syntech) and recorded on a PC using Syntech Autospike 32 software.

The antennal preparation was continuously flushed with charcoal filtered and humidified air (0.5 m/s, Syntech CS 55 Stimulus Controller) through glass tubing (10-mm ID) positioned 10 mm from the recording site and connected to the olfactory detection port of the GC. Synthetic reference or test stimuli were introduced 10 cm from the tubing outlet. Sensilla showing spontaneous spiking activity were screened for responses to full host plant headspace extracts loaded onto filter paper strips inserted into disposable Pasteur pipettes. If responses were detected, GC-SSR recordings were initiated.

Action potentials originating from OSNs within a sensillum were extracted as digital spikes from the analog signal using Syntech Auto Spike 32 software. Due to the difficulty in separating action potential (spike) amplitudes from colocated OSNs within a sensillum (ranging from 1 to 3 in basiconic and trichoid sensilla; Shields and Hildebrand 1999), all analyses were based on the total response from all OSNs within each sensillum. Instantaneous spike frequencies for all OSNs within a sensillum were recorded in 1 s bins for the entire GC run time. To be considered a significant excitatory or inhibitory response, the instantaneous spike frequency after solvent elution must be 4 standard deviation (SD) above or below the average of the first 100 s before solvent elution. Responses were then matched with the respective GC peak. If sensilla were still responsive after GC-SSR recording, they were stimulated with reference compounds in hexane tested at 1 µg loadings on filter paper strips (0.5 × 1 cm) in Pasteur pipettes delivered at 0.5 L/min for 0.5 s duration.

Sensillum responses were compared as a population using PASW 18.0 software. Number of sensilla responding per compound type was assessed using chi-square tests. Sensillum response profiles to extracts and synthetic stimuli were clustered using Ward's method with squared Euclidian distance. Species and condition-specific sensilla were

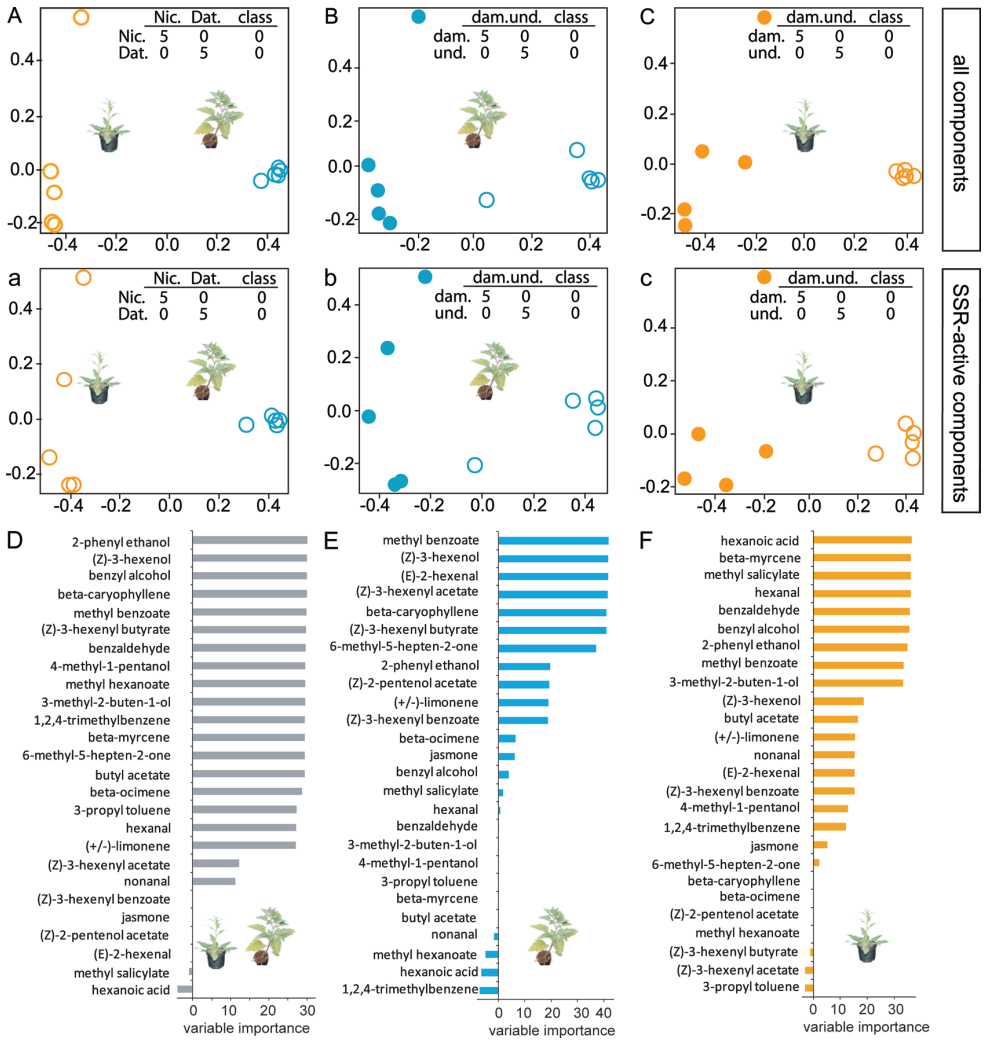

Figure 4 Host plant species and plant state can be separated by both volatile emission and GC-SSR response. (A–C) Metric multidimensional scaling representation of *Datura wrightii* and *Nicotiana attenuata* based on all volatiles found in headspace samples of the 2 host plants. (A) species representation, (B) and (C) comparison of headspace samples of undamaged (open circles) and larval-damaged (full circles) *D. wrightii* and *N. attenuata*, respectively. (a–c) The same type of analysis using SSR active compounds only. During 10 0000 replicated tree constructions samples were always assigned correctly (see inlet confusion matrices; Nic, *N. attenuata*; Dat, *D. wrightii*; dam, damaged; und, undamaged; class error, number of classification errors). (D–F) Contribution of different compounds to the separation of samples in (a–c). High absolute value of variable importance depicts strong contribution. This figure appears in color in the online version of *Chemical Senses*.

determined as sensilla responding to at least 1 compound that only elicited responses in the presence of a certain species or condition, but did not respond to compounds specific to other species or conditions.

Results

Oviposition preference for host species and condition

In the initial experiments with full access to the plants, *M. sexta* females deposited almost 6 times more eggs on *D. wrightii* plants (a total of 29.6 eggs per female ± 33.7 SD; Figure 2A) compared with *N. attenuata* (5.3 ± 7 SD; Figure 2A), whereas *P. parviflora* elicited an intermediate preference (24 ± 29.9 SD; Figure 2A) and the nonhost *B. oleracea* was not preferred (1.2 ± 2.7 SD; Figure 2A). Egg numbers were significantly different for *D. wrightii* and *P. parviflora* compared with *B. oleracea*, and for *D. wrightii* compared with *N. attenuata* (Friedman test, $P < 0.0001$; Dunn's Multiple Comparison test, $P < 0.05$). Among the host plants, *D. wrightii* and *N. attenuata* exhibited the clearest difference in oviposition preference and were thus chosen for further analyses. In subsequent experiments, gravid females also deposited significantly more (Figure 2B)

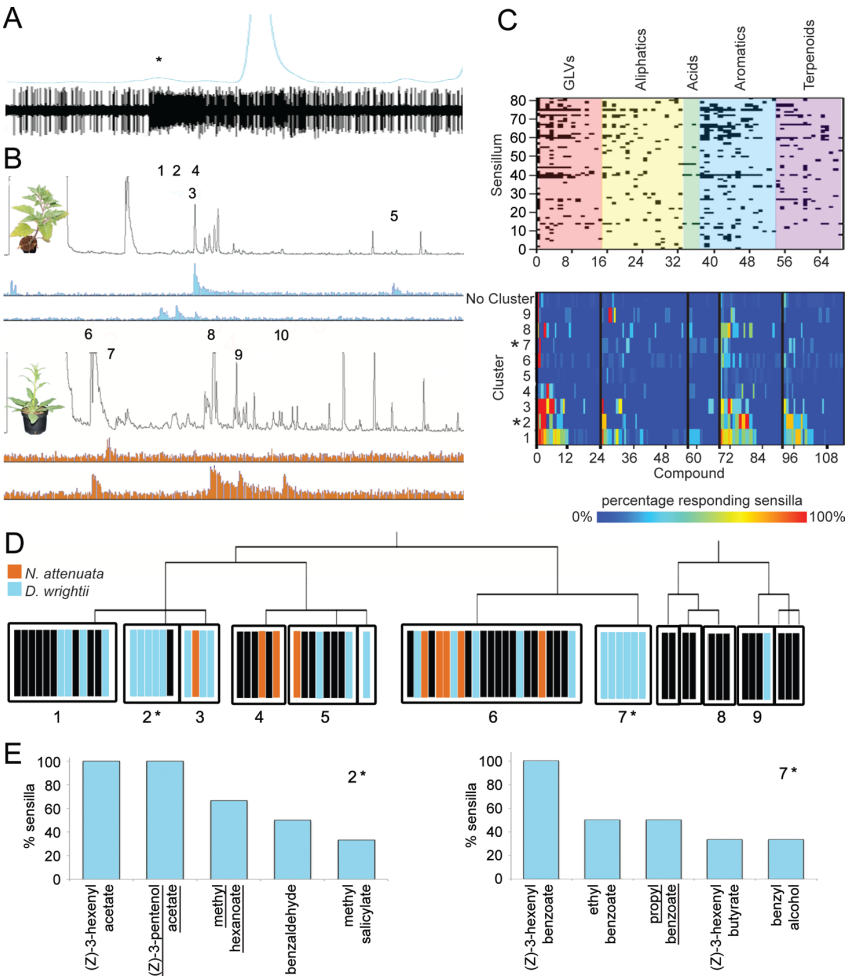

Figure 5 GC-single sensillum responses are both specific and broadly tuned. (A) Responses (t = 50 s) of a single sensillum to volatiles from feeding-damaged *Datura wrightii* headspace; * response to benzaldehyde. (B) Sample traces showing the specificity of response for 4 sensilla to an undamaged *D. wrightii* (top) or *Nicotiana attenuata* (bottom) extract, respectively. The responses (labeled with numbers) correspond to the following compounds: 1) methyl hexanoate, 2) benzaldehyde, 3) (Z)-3-hexenyl acetate, 4) (E)-2-hexenyl acetate, 5) α-farnesene, 6) 3-methyl-2-buten-1-ol, 7) 4-hydroxy-2-pentanone, 8) limonene, 9) methyl benzoate, and 10) methyl salicylate. (C, top) Response matrix for all 81 sensilla tested to plant and synthetic stimuli. The range of responses across the 5 main categories of compounds (GLVs, aliphatics, acids, aromatics, and terpenoids) shows the broad response profiles exhibited by many sensilla. Only compounds that elicited a response in at least 2 sensilla are shown. (C, bottom) Response matrix for the 9 clusters shown in (D). "No cluster" refers to the few sensilla in (C) that established clusters with maximum 2 sensilla. Color scale represents percentage of sensilla within the cluster that elicited a response to a particular compound from 0% to 100%. (D) Cluster analysis (Ward's method; squared Euclidian distance) for all 81 recorded sensilla (bars) shows the presence of species-specific clusters. Sensilla are labelled as *D. wrightii* specific or *N. attenuata* specific if they exhibited responses specific to 1 plant species. Black bars indicate nonspecific sensilla. (E) Response profiles of the 2 *D. wrightii* specific clusters in (D) (cluster 2 and 7). Both response profiles show only compounds common to 2 or more sensilla in the cluster. This figure appears in color in the online version of *Chemical Senses*.

eggs on tents housing *D. wrightii* (36 ± 69.2 SD) compared with *N. attenuata* (2.3 ± 6.1 SD, Wilcoxon signed-rank test, $P < 0.035$) precluding physical contact with the plants.

We also examined whether conspecific larval damage affected oviposition choice. We did not find any significant impact of larval damage on the oviposition preference of females on *D. wrightii* (number of eggs laid for undamaged, 97.6 ± 42.9 SD vs. damaged, 77.9 ± 31.42 SD, Wilcoxon signed-rank test, $P = 0.102$, Figure 2C, left). However, females deposited significantly more eggs on undamaged (74.8 ± 44.7 SD) versus larval-damaged *N. attenuata* plants (56.9 ± 30.6 SD, Wilcoxon signed-rank test, $P < 0.018$, Figure 2C, right). This reduction in oviposition was only detected in the presence of both larvae and feeding damage, rather than with either damage-specific plant or larval volatiles alone (see Figure 3A–D).

In addition, we observed the frequency of plant contacts for each of 10 gravid females choosing between damaged

and undamaged *N. attenuata* or *D. wrightii*, respectively. We observed no difference in the ratio of rejection after plant contact versus oviposition behavior (contacts resulting in abdomen curling) on either undamaged or damaged plants of both species (the odds ratio comparing conditions was 0.98 in *N. attenuata* and 1.18 in *D. wrightii* [Fisher's Exact test: *N. attenuata*, $P = 0.933$; *D. wrightii*, $P = 0.383$]). This suggests that physical contact with the plants does not affect choice between plant conditions in this species. Thus olfactory cues must play a primary role in the overall preference of *Manduca* for undamaged *N. attenuata*.

Chemical profile of host species and condition

Both species-specific and condition-specific compounds were detected in headspace samples from undamaged and feeding-damaged *N. attenuata* and *D. wrightii* plants (Figure 2D; Supplementary Table 1). The specificity of compounds was assessed in relation to the tested host species and conditions. Thus, the term "specific" refers to compounds that were emitted by *D. wrightii* but not by *N. attenuata* and vice versa or by undamaged plants only in comparison to larval-damaged plants. Compounds common to both species and/or states differed with respect to their proportion of the total blend (Figure 2D; Supplementary Table 1). Green leaf volatiles (GLVs) and their derivatives, terpenoids, and aromatic and aliphatic compounds all contributed to the characteristics of each blend. Overall, *N. attenuata* had significantly higher volatile emission rates (Figure 2D; total emission rate ± standard error: undamaged 10.5 ± 0.5 ng/min, feeding damaged 18.8 ± 1.4 ng/min) compared with *D. wrightii* (undamaged 1.8 ± 0.3 ng/min, feeding damaged 5.4 ± 1.2 ng/min).

We computed "random forest" classification trees (Breiman 2001; Ranganathan and Borges 2010) for complete volatile profiles of *D. wrightii* and *N. attenuata*. The analysis led to clear separation of *D. wrightii* and *N. attenuata* headspace samples (Figure 4A) as well as distinct clustering of feeding-damaged and undamaged samples in both species (Figure 4B,4C). When comparing headspace samples of differently induced *N. attenuata* plants, the volatile bouquet of all treatments also separated well from the headspace composition of control plants (Figure 3a–d).

Electrophysiological analysis of host species and condition

OSNs in a total of 81 *M. sexta* female olfactory sensilla were tested against headspace volatiles of damaged as well as undamaged *N. attenuata* and *D. wrightii* plants delivered via gas chromatography. The sensilla were also tested against synthetic standards (Supplementary Table 2). OSNs exhibited robust and discrete responses to compounds (Figures 2E and 5A,B). Sensilla thus demonstrated a number of different response profiles (Figure 5B,5C), although individual OSNs within single sensilla could not be unambiguously identified. Sensilla responded to a total of 119 natural headspace and synthetic compounds (Figure 2E; Supplementary Table 2). Overall, a significantly higher percentage of sensilla responded to GLV and aromatic compounds rather than aliphatics, acids, or terpenoids (Supplementary Table 2; Figure 2E; chi-square = 87.6, $n = 81$, $P < 0.001$). In 51% of the tested sensilla, more than 5 compounds elicited a response (Figure 5C, median = 6), whereas only 30% responded to 3 or fewer compounds.

When tested against headspace extracts, a higher percentage of sensilla responded to GLVs and aromatics from *D. wrightii*, whereas more sensilla responded to acids and aliphatics from *N. attenuata* (chi-square = 23.5, $n = 52$, *D. wrightii* and $n = 34$, *N. attenuata*, $P < 0.001$). Reducing the chemical data sets in the "random forest" analysis to the compounds found to be physiologically active in the GC-SSR experiments did not diminish the distinct grouping of headspace samples along physiological states or species (Figure 4a–c). The reduced set of SSR active compounds was thus sufficient to provide information on both species and state of the volatile source. A small number of compounds contributed to all 3 tested classifications of species or state (2-phenyl ethanol, (*Z*)-3-hexenol, benzyl alcohol, methyl benzoate, 6-methyl-5-hepten-2-one, hexanal, and (+/−) limonene; Figure 4D–F).

In addition, a number of responses to compounds present in only 1 of the 2 tested host species were found by GC-SSR (dashed sections of Figure 2D,2E). Sensilla with responses to *D. wrightii*-specific compounds also grouped into at least 2 separate clusters according to response profile (Figure 5D,5E). Conversely, sensilla with condition-specific responses (Figure 2E; compare filled vs. open bars) did not cluster according to response profile. In summary, the OSNs assessed here exhibit both broadly tuned and specific responses that allow discrimination of both host species and quality by volatile profile alone.

Discussion

The natural environment of an herbivorous insect consists of a number of plant species and conditions, which provide more or less suitable oviposition sites. We show that *M. sexta* females make a clear distinction between relevant species, and in some cases, between plants of differing condition, which translates into host quality in the field (Mira and Bernays 2002; Kessler and Baldwin 2004). Both host plant species and quality could be distinguished by volatile profile, and these volatiles were detected by an antennal periphery that exhibits both broadly tuned and specific responses to species and states. The set of sensilla recorded here detected more than half of the volatiles emitted by the 2 main host plants in this study, and the sensory arsenal present on the *Manduca* antenna is equipped to distinguish both host species and state. Building on the wealth of prior knowledge concerning *M. sexta* olfaction, we can now draw a direct

connection between behavioral preference, volatile release, and peripheral detection that has important implications for understanding the evolution of the olfactory system to meet the reproductive needs of a species.

The oviposition behavior of a female moth is a complex chain of decisions based on sensory input and the physiological state of the female (Yamamoto et al. 1969; Ramaswamy 1988). Here, the physiological state was kept as constant as possible, and the sensory input was compared. In our study, female moths made a choice between different species of unattacked potential host plants. *Datura wrightii* was the most accepted, *P. parviflora* received an (insignificantly) intermediate number of eggs, whereas *N. attenuata* was the least preferred. The *B. oleracea* nonhost—as expected—elicited almost no oviposition visits at all. When excluding direct contact with the host plant and providing the moths only with plant-derived olfactory cues, again the moths oviposited preferably on a net surrounding *D. wrightii* versus *N. attenuata*. As oviposition is the ultimate choice of the female, it represents a highly conservative measurement of host acceptance, and suggests the importance of olfactory input in female choice. However, without physical contact to the plants the rate of nonresponding females increased, indicating that contact and/or visual cues also contribute to the full sequence of egg laying behavior (Raguso and Willis 2005).

The olfactory-based species choice of *M. sexta* females can be placed into both an ecological and evolutionary context. *Datura wrightii* and *N. attenuata* grow side by side in Utah's Great Basin desert (Kessler and Baldwin 2002). They do, however, exhibit dramatically different life histories. *Datura wrightii* is a vigorous herbaceous perennial plant with large leaves and flowers, and of more or less constant yearly availability (Bronstein et al. 2009). It thus provides ample and predictable biomass for consumption by larvae. *Nicotiana attenuata*, on the other hand, is an annual plant with a basal rosette and erect slender stems supporting small flowers. It occurs in extreme numbers after bush fires, but is otherwise dormant in seed banks (Wells 1959). Each *N. attenuata* plant supports only a single larva (McFadden 1968; Kessler and Baldwin 2001), but when these plants occur they constitute a considerable resource. Consequently, when both plants are present, females may exhibit a preference for *D. wrightii*, but must be prepared to accept either species due to availability.

Previous field and chemical analyses with these 2 plant species suggest that *Manduca* exhibits a preference for unattacked *N. attenuata* in the field that could relate to its herbivore-specific induced volatiles (Baldwin et al. 2001; Kessler and Baldwin 2001). In contrast, the volatile profiles of *D. wrightii* do not depend on herbivore species (Hare and Sun 2011). Correspondingly, in our study, female moths exhibited a significant preference for nonattacked *N. attenuata* plants, whereas in *D. wrightii* they did not. This behavior also connects well to the already discussed idea, that females would be well advised to refrain from oviposition on the smaller, already attacked *N. attenuata*, whereas oviposition on the larger, though occupied *Datura* still might prove beneficial. Interestingly, only the combination of an attacked *Nicotiana* plant with the odor of larvae and their waste products provided a choice-eliciting stimulus in our behavioral assays (Kessler and Baldwin 2001). Such a combined stimulus indicates that not only has the plant been attacked but the attackers are still actively feeding.

How has the olfactory system evolved to meet these ecological needs? By recording from 81 individual sensilla responding to volatile emissions of *D. wrightii* and *N. attenuata*, we performed one of the largest GC-SSR studies so far, thus gathering chemical as well as detailed information on peripheral olfactory specificity to host plants. The chemical investigations revealed that both species and condition provide volatile signatures that could be clearly distinguished at the periphery (e.g., Figure 4). The female has an excellent olfactory opportunity to remotely utilize these patterns for her choice, without needing to contact the leaves. Yet, although both *Datura* and *Nicotiana* changed their volatile emissions drastically after attack, this change was only behaviorally relevant in the case of *Nicotiana*. Thus, having the sensory capability to make a choice does not mean that an animal always makes use of it.

The female sensilla investigated detected roughly 60% of all odorants produced by the plants, indicating that the antennal OSNs can detect a large portion of the volatile emissions emanating from these potential hosts. Given that the majority of OSNs were also broadly tuned to a number of the host volatiles, the odor "image" of these plants must in large part be relayed combinatorially to the central nervous system. Nevertheless, we also found that female *Manduca* possessed separate sensillum types with OSNs detecting compounds specific to only one of the tested host species, suggesting that host species can still be segregated at the antennal periphery. The peripheral coding of *D. wrightii* and *N. attenuata* odor emissions thus seems to contain 2 components; 1 ratiometric, with sensilla detecting molecules emitted by both species in different proportions, and 1 detecting species-specific odorants. This creates a sensory system in which both specific and broadly tuned OSNs have shared, but nonoverlapping response profiles: a "selectively nonselective" system (Alicia Anderson, APACE, Honolulu, HI, 2009).

An interesting comparison can be made between olfactory-based oviposition and nectar-feeding preference in *Manduca* as studied by Riffell et al. (2009). These authors elegantly showed that moths orient toward *D. wrightii* flowers, and that the volatile mixture emitted by the flower can be mimicked by a highly reduced blend. Although both antagonistic and mutualistic interactions can take place on the same plant, resulting in a typical trade-off situation (Bronstein et al. 2009), the selection pressures between oviposition and nectar feeding are very different. When a female moth looks for an oviposition site, it is vital that she optimizes her choice. Choosing a poor host for her offspring may dramatically

reduce the genetic output in the next generation. In contrast, the search for a nectar-rich flower is not under immediate selection pressure, and it is possible to repeat the search expending only time and energy. In addition, the search for a nectar source is more or less always a search for a reward, so both the feeding moth and the pollinated flower are interested in a successful outcome. Simple and unambiguous signals, for example, odor constancy (Raguso 2008) will therefore be favored over evolutionary time. Correspondingly, a widely reduced and physiologically active blend of flower volatiles attracts foraging hawk moths (Riffell et al. 2009). Conversely, host search is antagonistic; the moth female wants to locate a host plant, which naturally benefits by escaping the fate of being consumed. A stronger crypsis in plant odor emissions should thus be favored, creating a stronger selection pressure on the moth olfactory system to detect these kairomones. In parallel, however, such pressures might be counteracted by physiological processes directly involved in plant defense against *Manduca* moths and other potential herbivores (Dicke and Baldwin 2010).

Our study indicates that female *M. sexta* make an adapted choice of host plants based on olfactory information, and that the olfactory periphery is equipped to discriminate different species and qualities. Nevertheless, visual and contact cues may also provide additional information to host searching insects (Stenberg and Ericson 2007; Städler and Reifenrath 2009; Kuehnle and Mueller 2011). Lower response rates in our experiment with obstructed visual and contact stimuli indicate that these cues also play a role for the ovipositing *M. sexta* female. Herbivory by a single species is also quite unnatural, and volatile emission from the simultaneous feeding of different guilds cannot be anticipated from herbivore-specific plant responses (Delphia et al. 2007). Further investigations should dissect responses to more "natural" plants, having been attacked by a host of herbivores, bacteria, fungi, and other natural enemies. Another important aspect is the flexibility of the system. To what degree can the female choice of host plant be modified through experience? Attraction to *Agave* flowers is not innate, but has to be learned, unlike *Datura* (Riffell et al. 2008). Similarly, adult experience can shape oviposition behavior in Lepidoptera (Tammaru and Javois 2005; Olsson et al. 2006) as well as other insect taxa (Jaenike 1983; Kaur et al. 2003). Corresponding investigations in ovipositing *Manduca* necessitate field studies to properly simulate natural conditions. A deeper understanding of higher levels of olfactory processing, that is, in the antennal lobe, mushroom body, and lateral horn, is also crucial to further increase our understanding of how the female decodes kairomone signals into attraction or nonattraction.

Conclusions

We show that female *M. sexta* sphingid moths display an odor-dependent oviposition preference between different ecologically relevant host species, and also between herbivore-attacked and -nonattacked plants. Combined chemical and physiological analysis of 2 of these host plants, *D. wrightii* and *N. attenuata*, show that both host plant species and quality can be distinguished by volatile profile, and that these volatiles are detected by an antennal periphery that exhibits both broadly tuned, and specific responses to species and states. Out of more than 100 compounds identified, more than 60% were detected by the OSNs measured in this study. Our findings suggest that the odor image of a host is already represented at the sensory periphery, and that distinct olfactory activation patterns for both host species and quality are conveyed to the brain, allowing the female moth to distinguish both host species and quality. We can now draw a direct connection between behavioral preference, volatile release, and peripheral detection that highlights the important role of olfactory cues in *M. sexta* oviposition choice.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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CHAPTER II

Feeding-induced rearrangement of green leaf volatiles reduces moth oviposition

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RESEARCH ARTICLE

Feeding-induced rearrangement of green leaf volatiles reduces moth oviposition

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Abstract The ability to decrypt volatile plant signals is essential if herbivorous insects are to optimize their choice of host plants for their offspring. Green leaf volatiles (GLVs) constitute a widespread group of defensive plant volatiles that convey a herbivory-specific message via their isomeric composition: feeding of the tobacco hornworm *Manduca sexta* converts (*Z*)-3- to (*E*)-2-GLVs thereby attracting predatory insects. Here we show that this isomer-coded message is monitored by ovipositing *M. sexta* females. We detected the isomeric shift in the host plant *Datura wrightii* and performed functional imaging in the primary olfactory center of *M. sexta* females with GLV structural isomers. We identified two isomer-specific regions responding to either (*Z*)-3- or (*E*)-2-hexenyl acetate. Field experiments demonstrated that ovipositing *Manduca* moths preferred (*Z*)-3-perfumed *D. wrightii* over (*E*)-2-perfumed plants. These results show that (*E*)-2-GLVs and/or specific (*Z*)-3/(*E*)-2-ratios provide information regarding host plant attack by conspecifics that ovipositing hawkmoths use for host plant selection.
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Introduction

Insects rely on olfaction in most aspects of life: volatile signals guide them to food sources, mating partners and oviposition hosts. Especially for herbivorous insects, plant volatiles provide important cues to locate and identify appropriate host plants for their offspring. Upon herbivory, plants respond with an increased release and de novo synthesis of several volatile compounds from their vegetative tissues (***Mumm and Dicke, 2010***). These so-called herbivore induced plant volatiles can provide significant information to the surrounding environment as composition and abundance reflect several biotic and abiotic factors (***Takabayashi et al., 1995***; ***De Moraes et al., 1998***; ***Gouinguené et al., 2001***; ***Schuman et al., 2009***; ***Hare, 2010***).

Due to the context dependent composition of plant volatile signals, the ability to detect and discriminate volatile compounds is crucial for insects to generate appropriate behavioral responses. In insects and more specifically in the hawkmoth *Manduca sexta* (Lepidoptera/Sphingidae), olfactory sensory neurons (OSNs) located on the antennae detect odorant molecules (***Kalinová et al., 2001***; ***Shields and Hildebrand, 2001***; ***Fraser et al., 2003***; ***Spaethe et al., 2013***) and convey this information to the antennal lobe (AL), the first olfactory processing center. The AL of *M. sexta* females consists of about 70 structural and functional subunits called olfactory glomeruli (***Grosse-Wilde et al., 2011***). OSNs expressing the same receptor, and thus responding to the same set of odorants, converge onto the same glomerulus in the AL (***Gao et al., 2000***; ***Vosshall, 2000***) as has been demonstrated for *Drosophila melanogaster* and indirectly also in several moth species (***Hansson, 1997***). Spatio-temporal patterns of neuronal activity representing sensory input to the AL can be visualized by optical imaging methods (***Hansson et al., 2003***; ***Skiri et al., 2004***; ***Carlsson et al., 2005***; ***Silbering and Galizia, 2007***) enabling identification of compound- and blend-specific responses in the AL of *M. sexta* (***Hansson et al., 2003***; ***Bisch-Knaden et al., 2012***; ***Kuebler et al., 2012***).

eLife digest Plants have developed a variety of strategies to defend themselves against herbivorous animals, particularly insects. In addition to mechanical defences such as thorns and spines, plants also produce compounds known as secondary metabolites that keep insects and other herbivores at bay by acting as repellents or toxins. Some of these metabolites are produced on a continuous basis by plants, whereas others—notably compounds called green-leaf volatiles—are only produced once the plant has been attacked. Green-leaf volatiles—which are also responsible for the smell of freshly cut grass—have been observed to provide plants with both direct protection, by inhibiting or repelling herbivores, and indirect protection, by attracting predators of the herbivores themselves.

The hawkmoth *Manduca sexta* lays its eggs on various plants, including tobacco plants and devil's claw plants. Once the eggs have hatched into caterpillars, they start eating the leaves of their host plant, and if present in large numbers, these caterpillars can quickly defoliate and destroy it. In an effort to defend itself, the host plant releases green-leaf volatiles to attract various species of *Geocoris*, and these bugs eat the eggs.

One of the green-leaf volatiles released by tobacco plants is known as (*Z*)-3-hexenal, but enzymes released by *M. sexta* caterpillars change some of these molecules into (*E*)-2-hexenal, which has the same chemical formula but a different structure. The resulting changes in the 'volatile profile' alerts *Geocoris* bugs to the presence of *M. sexta* eggs and caterpillars on the plant.

Now Allmann et al. show that adult female *M. sexta* moths can also detect similar changes in the volatile profile emitted by devil's claw plants that have been damaged by *M. sexta* caterpillars. This alerts the moths to the fact that *Geocoris* bugs are likely to be attacking eggs and caterpillars on the plant, or on their way to the plant, so they lay their eggs on other plants. This reduces competition for resources and also reduces the risk of newly laid eggs being eaten by predators. Allmann et al. also identified the neural mechanism that allows moths to detect changes in the volatile profile of plants—the *E*- and *Z*- odours lead to different activation patterns in the moth brain.

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Green leaf volatiles (GLVs) constitute a large group of herbivore-induced plant volatiles characterized by a C6-backbone. While emitted only in trace amounts from healthy, undamaged plant tissue, they are emitted instantly after cell disruption (***Turlings et al., 1995***; ***D'Auria et al., 2007***). GLVs are generated from C18-fatty acids via the enzymes lipoxygenase (LOX) and hydroperoxide lyase (HPL; ***Allmann et al., 2010***). One of the most abundant GLVs, (*Z*)-3-hexenal, originates from the cleavage of α-linolenic acid through the activity of HPL and it partly rearranges to (*E*)-2-hexenal. Both alkenals can be further metabolized by an alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT; ***D'Auria et al., 2007***) to the corresponding alcohols and their esters (***Matsui, 2006***).

GLVs have been assigned various plant defense-associated functions by directly inhibiting phytopathogens (***Hamilton-Kemp et al., 1992***; ***Nakamura and Hatanaka, 2002***; ***Prost et al., 2005***) and repelling several herbivore species (***De Moraes et al., 2001***; ***Kessler and Baldwin, 2001***; ***Vancanneyt et al., 2001***; ***Zhang and Schlyter, 2004***). Remarkably, GLVs also function as indirect plant defenses by attracting foraging predators and host-seeking parasitoids to the plant and its attacker (***Kessler and Baldwin, 2001***; ***Shiojiri et al., 2006***; ***Halitschke et al., 2008***; ***Schuman et al., 2012***) reminiscent of the role of other herbivore induced plant volatiles.

Due to their ubiquity and instant release, GLVs are thought to act as nonspecific signals of plant damage (***Hatanaka et al., 1987***; ***Hoballah et al., 2002***). We recently showed that an enzymatic component of the oral secretions (OS) of *M. sexta* larvae adds an herbivory-specific feature to the GLV signal. Mechanically damaged leaves of *Nicotiana attenuata* released large amounts of (*Z*)-3-GLVs and low amounts of (*E*)-2-GLVs. However, when the plant was attacked by *M. sexta* caterpillars or when puncture wounds of plant leaves were treated with *M. sexta's* OS, the amount of (*E*)-2-GLVs released increased, while the amount of (*Z*)-3-GLVs decreased, resulting in a distinct change in the (*Z*)-3/(*E*)-2-ratio of GLV emissions. This herbivore-induced change in the (*Z*)-3/(*E*)-2-ratio attracted the generalist hemipteran predator *Geocoris* spp., which decreased the herbivore load on the plant by feeding on herbivore eggs (***Allmann and Baldwin, 2010***).

Our discovery of a (3*Z*):(2*E*)-enal isomerase in the OS of *M. sexta* larvae raises many questions. Why does *Manduca* produce an enzyme that generates volatiles which betray the insect to its enemies, and why did evolution not select against this isomerase? The enzyme might be maladaptive and therefore is, or will be, under negative selection. The occurrence of this specific isomerase activity in at least two other lepidopteran species (**Allmann and Baldwin, 2010**) however, suggests that it may have a beneficial function that outweighs the larva's net costs of maintaining such an enzyme. It is well known that plants exchange information above ground by releasing volatiles into the air (**Baldwin, 2010**), which can be perceived by insects as well. Insects can use plant derived volatiles for communication by giving the herbivore induced volatile blend a 'personal' note—in our case, by converting (*Z*)-3-GLVs to their structural isomers and by changing the (*Z*)-3/(*E*)-2 ratio. Which message could *M. sexta* larvae thereby communicate? In this study we hypothesized that the altered GLV emission might serve to reduce the number of competitors on their host plant by informing conspecific ovipositing moths that this plant is already occupied and, possibly, receiving increased predation. Reduced oviposition of *Manduca* moths in response to feeding damage has been shown in field experiments with *M. quinquemaculata* (**Kessler and Baldwin, 2001**) as well as under laboratory conditions with *M. sexta* (**Spaethe et al., 2013**). Deviating from the previous study, we chose *Datura wrightii* (Solanaceae) for our experiments. *Datura* is a highly preferred host plant of both *M. sexta* and the congeneric *M. quinquemaculata* for both nectar feeding (**Alarcón et al., 2008**; **Kessler, 2012**) and oviposition (**Spaethe et al., 2013**). Its distribution covers southwestern USA (**Avery, 1959**; **Munz, 1973**) overlapping with the occurrence of both *Manduca* species. The perennial shrub is repeatedly described to quickly regrow leaves after herbivore damage (**Bronstein et al., 2009**; **Reisenman et al., 2010**, **2013**). Laboratory experiments failed to find reduced oviposition on damaged *D. wrightii* (**Reisenman et al., 2013**; **Spaethe et al., 2013**) suggesting flexibility in oviposition choice of *Manduca* females. As the previously examined *N. attenuata* (**Gaquerel et al., 2009**), *D. wrightii,* respond to *Manduca* herbivore attack by emitting GLVs (**Hare and Sun, 2011**). While we investigated GLV emission during the day when focusing on the diurnal egg predator *Geocoris* ssp., *Manduca* moths oviposit at twilight and night (**Madden and Chamberlin, 1945**; **Lingren et al., 1977**). Therefore, we decided to collect volatiles during these times instead. We expected the shift to occur also during the night, as in several plant species GLV emission has been shown to occur also in the dark period (**Loughrin et al., 1994**; **Arimura et al., 2008**), and the respective shift in the (*Z*)-3/(*E*)-2-ratio is caused by *M. sexta* oral secretions and not by the plant itself (**Allmann and Baldwin, 2010**). However, volatile emissions vary with light regime (**Halitschke et al., 2000**; **De Moraes et al., 2001**; **Gouinguene and Turlings, 2002**; **Morker and Roberts, 2011**), and we therefore chose two nocturnal light conditions differing by moonlight intensity to examine whether light intensity affects GLV emission in *D. wrightii*. We performed functional imaging in the antennal lobe of female *M. sexta* moths asking whether (*Z*)-3- and (*E*)-2-structural isomers of any of the tested GLVs can be discriminated by the olfactory system. In classical host recognition experiments the Colorado potato beetle *Leptinotarsa decemlineata* has been shown to recognize and avoid altered ratios of (*Z*)-3- and (*E*)-2-GLVs emitted by its host plant *Solanum tuberosum* (**Visser and Avé, 1978**). Furthermore, enantioselectivity has been reported for projection neurons in the female AL of *M. sexta* in response to (+)- and (−)-linalool (**Reisenman et al., 2004**). Thus, we hypothesized that *M. sexta* females would be able to differentiate between (*Z*)-3 and (*E*)-2-isomers of at least one GLV. If so, ovipositing *M. sexta* should avoid plants with increased levels of (*E*)-2-GLVs as they indicate host plants with increased larval feeding competition and predation risk (**Allmann and Baldwin, 2010**). Here we show by combining field studies with neurophysiological imaging techniques that (i) OS-induced *D. wrightii* plants have altered (*Z*)-3/(*E*)-2-ratios also during the night under both laboratory and field conditions, (ii) *Manduca* females detect and discriminate the (*Z*)-3- and (*E*)-2-isomers and (iii) show ovipositional preference for high (*Z*)-3/(*E*)-2-GLV ratios.

Results

Application of *M. sexta* OS to leaf wounds triggers pronounced changes in the GLV profile of *Datura wrightii*

To investigate whether application of *M. sexta's* OS onto wounded leaves of *Datura wrightii* plants causes a similar shift in the (*Z*)-3/(*E*)-2-ratio as observed in *Nicotiana attenuata*, we compared the emissions of mechanically wounded *D. wrightii* plants that were treated with either water as a control (w + w) or with *M. sexta's* OS (w + OS) in growth chamber experiments. During the day, application of

OS onto wounds caused a significant decrease in the (*Z*)-3/(*E*)-2-ratio of the GLVs released from *Datura* plants compared with control plants (***Figure 1A***, day).

Since *Manduca* moths are crepuscular and nocturnal insects (***Theobald et al., 2010***), we repeated the experiment under low light and no-light conditions to mimic sunset and night (***Figure 2***). The (*Z*)-3/(*E*)-2-ratio of the aldehydes differed significantly between treatments also at sunset and night light intensities (***Figure 1A***, sunset). However, the (*Z*)-3/(*E*)-2-ratio of w + w treated plants also decreased with decreasing light intensities, which was mainly caused by increased (*E*)-2-hexenal emissions (***Figure 3*** and ***Table 1***). Correspondingly, treatment-dependent differences in (*Z*)-3/(*E*)-2-ratios for the alcohol and the hexenyl acetate decreased under lower light conditions and were not found during the night (***Figure 1A***, sunset, night).

To evaluate whether w + w and w + OS treated plants release GLVs in distinguishable ratios under normally variable conditions found in nature, we trapped volatiles during daylight and repeatedly at night from a native *D. wrightii* population in the Utah desert during the 2011 field season. We

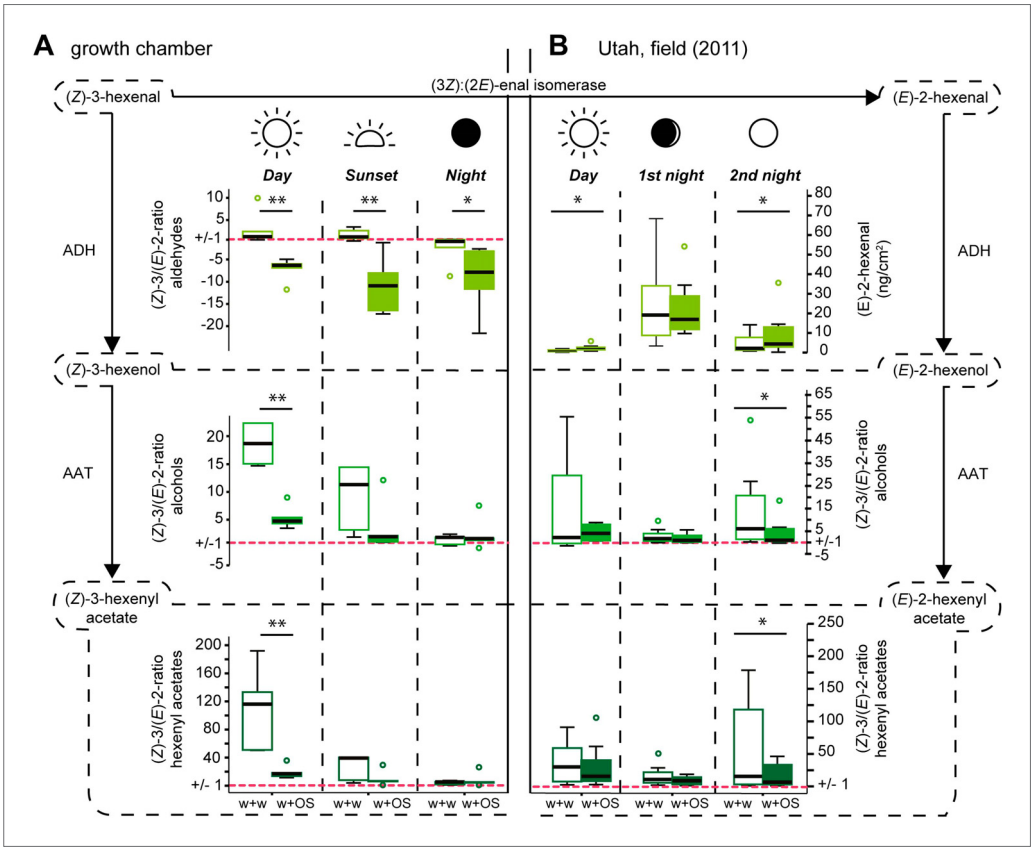

Figure 1. Diurnal changes in the emitted (*Z*)-3/(*E*)-2-ratios of GLVs in *Datura wrightii* plants. (*Z*)-3/(*E*)-2-ratios of GLVs in *Datura wrightii* plants represented as box plots. (**A**) Growth chamber experiment: a single not yet fully developed leaf of each *D. wrightii* plant was mechanically wounded and treated with water (w + w) or *M. sexta* OS (w + OS) during three different light conditions to mimic day, sunset, and night. (**B**) Field experiment: Three single previously undamaged leaves per plant were chosen and randomly assigned to a treatment (control, w + w or w + OS). Values of the control leaf were subtracted from the values of treated leaves. As (*Z*)-3-hexenal was not detectable in any of the field samples (*E*)-2-hexenal values are displayed in ng*cm^{-2}*2h^{-1} (adsorbents used in field collection are not accountable for the absence of (*Z*)-3-hexenal; ***Table 6***). For visual simplifications (*Z*)-3/(*E*)-2-ratios <1 are represented as their negative reciprocal. Values above '1' (red dotted line) thus represent treatment-groups that produced more of the (*Z*)-3-isomer and values below '1' represent treatment-groups that produced more of the (*E*)-2-isomer. Asterisks indicate significant differences between treatments (**A**: Mann–Whitney U test, **p≤0.01, *p≤0.05; n = 5), (**B**: Wilcoxon signed-rank test, *p<0.05; n = 8). ADH: alcohol dehydrogenase; AAT: alcohol acyl-transferase. The median is represented as a line in each box, box outlines mark the 25% and 75% percentiles; outliers are depicted as circles (if value > 1.5× the interquartile range). For raw data, see F1AB_AllmannSpaethe2012_volatiles.xlsx (Dryad: ***Allmann et al., 2012***).
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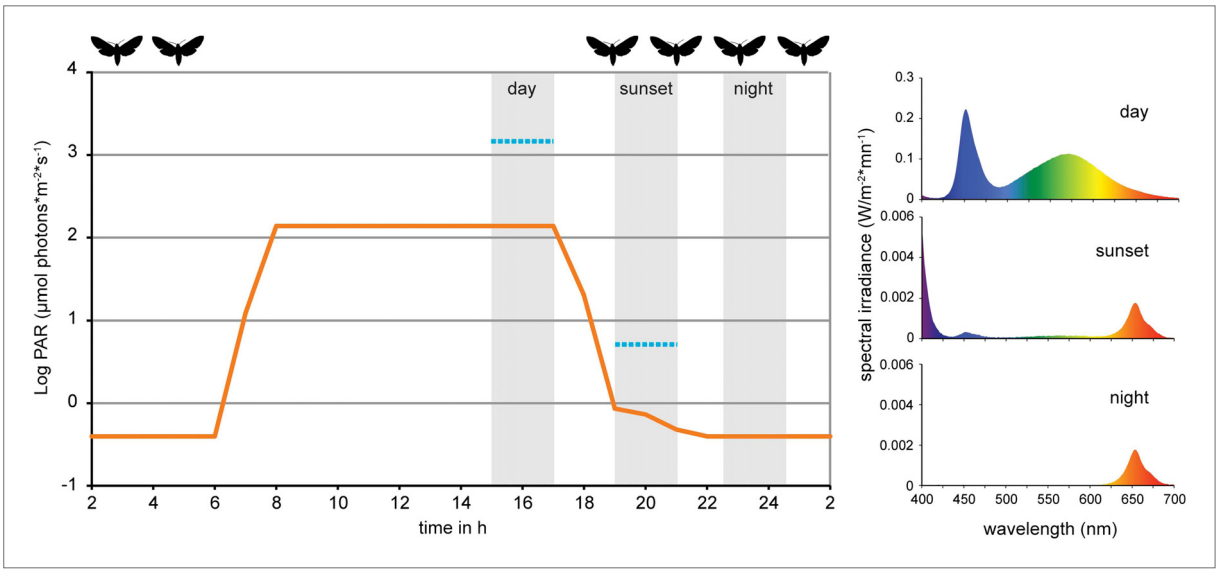

Figure 2. Light conditions during laboratory volatile collection. Light composition and intensity changed within 24 hr to simulate day, sunset and night condition. Photosynthetically active radiation (PAR, µmol photons*m^{-2}*s^{-1}, orange line) was measured for every light composition and ranged from 0.39 ± 0.01 SE at night to 138.37 ± 0.09 SE at full day conditions. Blue lines denote PAR values measured in the field during the respective volatile collection event (during the night samplings, PAR was below detection limit). For the graph values were logarithmized. Grey areas denote volatile collection events; respective light spectra are shown on the right. For representational reasons time scale starts at 2 am. Flight activity, related to nectar feeding and oviposition (***Madden and Chamberlin, 1945***; ***Lingren et al., 1977***), is indicated on top of the graph. For raw data, see F2_AllmannSpaethe2012_light.xlsx (Dryad: ***Allmann et al., 2012***).

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performed the experiments on two different days using eight plants for each sampling. Three equally sized leaves of each plant were selected and randomly assigned to one of the treatments (control, w + w or w + OS). Similar to previous experiments with *N. attenuata* (***Allmann and Baldwin, 2010***) we were unable to detect (*Z*)-3-hexenal in any of the samples.

During the day the application of OS to the wounds caused a significant increase in (*E*)-2-hexenal emissions compared with w+w treated leaves (***Figure 1B***, day, and ***Table 2***, day). As seen from the climate chamber experiment, average (*Z*)-3/(*E*)-2-ratio of the hexenyl acetates decreased (***Figure 1B***, day), but this change was not significant.

During the first night-experiment (first night, average temperature 17.6 ± 0.7°C, wind speed 1.1 ± 0.8 m/s, waxing crescent lunar illumination with 9% of the moon illuminated), plants of both treatments released very high but similar amounts of (*E*)-2-hexenal, and the (*Z*)-3/(*E*)-2-ratios of the alcohols and hexenyl acetates were low, but did not differ between treatments, resembling the results of the night trapping in the growth chamber (***Figure 1B***, first night, and ***Table 2***, first night).

During the second experiment (second night; average temperature 24.6 ± 0.8°C, wind speed 0.7 ± 0.8 m/s, full moon), approximately 2 weeks later, w + OS-treated plants released significantly higher amounts of (*E*)-2-hexenal (twofold increase compared with w + w treated plants) and the (*Z*)-3/(*E*)-2-ratios of the hexenols and hexenyl acetates were significantly lower compared with mechanically wounded plants that were treated with water only (***Figure 1B***, second night).

(*Z*)-3- and (*E*)-2-GLVs evoke different activation patterns in the antennal lobes of *Manduca sexta*

To evaluate if female *M. sexta* moths are physiologically able to discriminate between (*Z*)-3- and (*E*)-2-GLVs and between different (*Z*)-3/(*E*)-2-ratios we performed functional calcium imaging in the antennal lobes (AL) of females. Odor-evoked calcium changes in response to exposure to the pure (*E*)-2- and (*Z*)-3-isomers of hexenal, hexenol and hexenyl acetate led to activity in discrete regions corresponding to specific glomeruli in the AL of *M. sexta* females (***Figure 4A,B***). Aldehyde and alcohol structural isomers activated one single specific region (region of interest 2 [ROI 2], green), with significantly

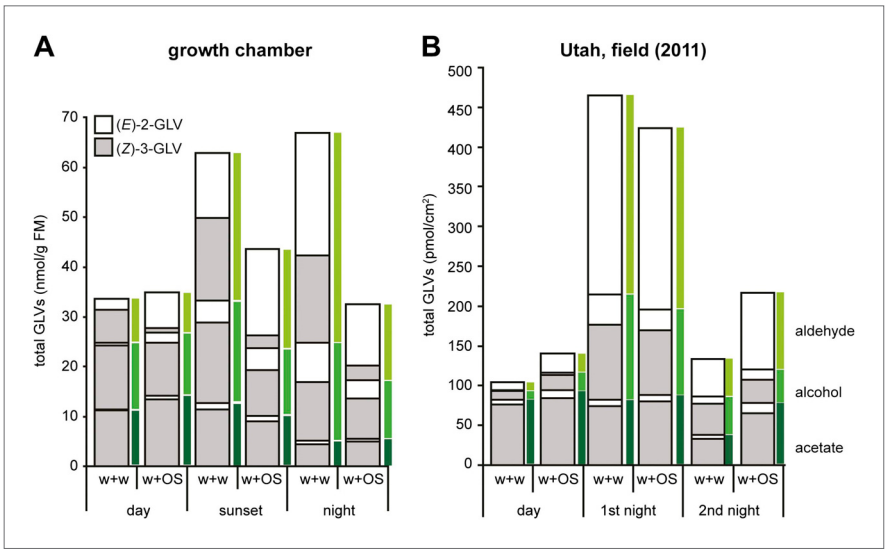

Figure 3. Total amounts of GLVs released from *Datura wrightii* plants at different times of the day in laboratory and field experiments. Mean release of major GLVs from *Datura wrightii* plants at different times of the day and at different light intensities. Grey and white bars represent (*Z*)-3- and (*E*)-2-GLVs, respectively. Single leaves were mechanically damaged and volatiles were trapped for 2 hr immediately after wounds had been treated with either water (w + w) or with *M. sexta's* OS (w + OS). (**A**) GLV emissions of *D. wrightii* plants under controlled light conditions in a growth chamber. Light conditions are explained in this figure. Quantities are given in nmol/g fresh mass (FM)/2 hr; n = 5. (**B**) GLV emissions of *D. wrightii* plants naturally grown in the field. Quantities are given in pmol/cm²/2 hr; n = 8. For an approximate comparison between (**A**) and (**B**): 50 cm² leaf area ≈ 1 g FM. Colored bars mark the emission of aldehydes (light green), alcohols (green) and acetates (dark green). For raw data, see F1AB_AllmannSpaethe2012_volatiles.xlsx (Dryad: ***Allmann et al., 2012***).

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stronger responses to the (*E*)-2- compared with (*Z*)-3-isomers (***Figure 4B***). (*Z*)-3-hexenyl acetate and its structural isomer activated three different regions in the female AL: a significantly (*Z*)-3-specific (ROI 3, blue), a significantly (*E*)-2-specific (ROI 4, pink) and an isomer-unspecific region (ROI 1, grey, ***Figure 4B***). The differences in activation patterns caused by stimulations with (*Z*)-3- or (*E*)-2-hexenyl acetate (***Figure 4C***) strongly suggest that the two odors activated OSNs expressing different sets of odorant receptor types on the female antennae. Of all tested GLVs, hexenyl acetate was the only compound eliciting isomer-specific responses in the AL, therefore we focused on (*Z*)-3- and (*E*)-2-hexenyl acetate for all further experiments.

As plants do not emit isomerically pure odors but rather mixtures, we studied AL representation of the acetate structural isomers in more detail by stimulating the antenna with blends of (*Z*)-3- and (*E*)-2-hexenyl acetate in different ratios (given as Z/E: 100/0, 80/20, 50/50, 20/80, 0/100). In ROI 3 (blue) calcium signals evoked by (*Z*)-3-hexenyl acetate-containing mixtures were significantly higher compared with stimulations with pure (*E*)-2-hexenyl acetate, which in turn did not differ from the mineral oil control (***Figure 5A,B***). For the (*E*)-2-specific ROI 4 (pink) stimulation with pure (*Z*)-3-hexenyl acetate led to significantly lower calcium responses when compared with pure (*E*)-2-hexenyl acetate and the 20/80 ratio, but was not different from stimulation with mineral oil (***Figure 5B***). Calcium responses of the unspecific ROI 1 (in grey) did not differ between the structural isomers and their mixtures (***Figure 5B***).

When comparing odor-evoked activation by different (*Z*)-3/(*E*)-2-ratios in ROI 3 and 4, stimulations with pure structural isomers as well as the 20% (*Z*)-3/80% (*E*)-2 mixture led to significantly different levels of neural activity in these (*E*)-2/(*Z*)-3-specific regions (***Figure 6A***). Activation patterns differed significantly for pure (*E*)-2-hexenyl acetate compared with the 50/50 and 80/20 (*Z*)-3/(*E*)-2 mixtures as well as for pure (Z)-3-hexenyl acetate compared with the 20/80 mixture (***Figure 6A***). However, no differences were found between the isomeric mixtures (20/80; 50/50; 80/20).

Table 1. GLV emission of *Datura wrightii* plants in the growth chamber during the first 2 hr after w + w or w + OS treatment with 100% light (day), 20–10% light (sunset) or 0% light (night)

				volatile release in µg / g leaf fresh mass	
	Class	**Common name**	**RT**	**w + w**	**w + OS**
Day	Aldehyde	(*Z*)-3-hexenal	8.54	0.64 ± 0.293	0.097 ± 0.027
		(*E*)-2-hexenal	10.49	0.22 ± 0.109	0.7 ± 0.17
	Alcohol	(*Z*)-3-hexenol	14.98	1.30 ± 0.511	1.06 ± 0.275
		(*E*)-2-hexenol	15.57	0.058 ± 0.034	0.195 ± 0.034
	Hexenylester	(*Z*)-3-hexenyl acetate	13.28	1.59 ± 0.442	1.92 ± 0.244
		(*E*)-2-hexenyl acetate	13.75	0.017 ± 0.004	0.105 ± 0.018
		(*Z*)-3-hexenyl butyrate	17.07	0.028 ± 0.009	0.051 ± 0.016
		(*E*)-2-hexenyl butyrate	17.44	0.01 ± 0.002	0.017 ± 0.004
Sunset	Aldehyde	(*Z*)-3-hexenal	8.54	1.62 ± 0.5	0.26 ± 0.118
		(*E*)-2-hexenal	10.49	1.28 ± 0.775	1.69 ± 0.697
	Alcohol	(*Z*)-3-hexenol	14.98	1.62 ± 0.433	0.93 ± 0.308
		(*E*)-2-hexenol	15.57	0.45 ± 0.315	0.44 ± 0.183
	Hexenylester	(*Z*)-3-hexenyl acetate	13.28	1.62 ± 0.431	1.28 ± 0.511
		(*E*)-2-hexenyl acetate	13.75	0.18 ± 0.12	0.158 ± 0.067
		(*Z*)-3-hexenyl butyrate	17.07	0.039 ± 0.011	0.031 ± 0.003
		(*E*)-2-hexenyl butyrate	17.44	0.013 ± 0.004	0.01 ± 0.001
Night	Aldehyde	(*Z*)-3-hexenal	8.54	1.71 ± 0.732	0.28 ± 0.118
		(*E*)-2-hexenal	10.49	2.43 ± 0.521	1.22 ± 0.697
	Alcohol	(*Z*)-3-hexenol	14.98	1.18 ± 0.35	0.81 ± 0.308
		(*E*)-2-hexenol	15.57	0.79 ± 0.14	0.37 ± 0.183
	Hexenylester	(*Z*)-3-hexenyl acetate	13.28	0.63 ± 0.268	0.71 ± 0.511
		(*E*)-2-hexenyl acetate	13.75	0.093 ± 0.04	0.083 ± 0.067
		(*Z*)-3-hexenyl butyrate	17.07	0.036 ± 0.002	0.033 ± 0.003
		(*E*)-2-hexenyl butyrate	17.44	0.01 ± 0.002	0.014 ± 0.001

Mean (±SEM; n = 5) release of GLVs in *D. wrightii* plants. A single not yet fully developed leaf of each plant was mechanically wounded and treated with water (w + w) or *M. sexta* OS (w + OS) during the day (**A**, 100% light), sunset (**B**, 20–10% light) and night (**C**, 0% light). Volatiles are listed by chemical classes and in order of their retention time.

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In addition to the isomer-specificity for both hexenyl acetates, ROI 3 and 4 displayed different response characteristics (***Figure 6B***). The level of activation of the (*Z*)-3-hexenyl acetate-specific ROI 3 (x-axis) was solely dependent on the presence of the (*Z*)-3-isomer and did not change with various amounts of it in the isomeric mixtures (ranging from 50 ng in 20/80 to 250 ng in 100/0). In contrast, the calcium signal in ROI 4 (y-axis) increased gradually with increasing percentage of the (*E*)-2-isomer up to 80% in the isomeric mixtures. Thus, ROI 4 is able to convey information about the ratio of (*Z*)-3- and (*E*)-2-hexenyl acetate in a mixture.

(*Z*)-3- and (*E*)-2-GLVs elicit different behavioral responses in ovipositing *Manduca* moths in nature

To test whether female *Manduca* moths use the herbivory-specific shift in (*Z*)-3/(*E*)-2-ratio to choose appropriate host plants for their offspring, we performed oviposition assays in the field during the 2010 field season (***Figure 7A***). We selected two native populations of *D. wrightii* plants located close to the Lytle Preserve research station (Santa Clara, UT). On each experimental day we tested two mixes that contained either only (*Z*)-3 or only (*E*)-2-GLVs or both structural isomers but in different ratios. Since our calcium imaging data suggested that *M. sexta* possesses (*Z*)-3- and (*E*)-2-hexenyl

Table 2. GLV emission of native *Datura wrightii* plants in the field (2011) during the first 2 hr after w + w or w + OS treatment; during day (1:30–3:30 pm), first or second night (0–2 am)

				Volatile release in ng/cm² leaf		
	Class	Common name	RT	Control	w + w	w + OS
Day	Aldehyde	(*E*)-2-hexenal	10.87	0.062 ± 0.006	1.02 ± 0.233	2.43 ± 0.597
	Alcohol	(*Z*)-3-hexenol	15.38	0.137 ± 0.067	1.21 ± 0.28	2.07 ± 0.465
		(*E*)-2-hexenol	15.97	0.248 ± 0.035	0.368 ± 0.088	0.57 ± 0.148
	Hexenylester	(*Z*)-3-hexenyl acetate	13.66	0.26 ± 0.083	11.1 ± 1.881	12.3 ± 2.067
		(*E*)-2-hexenyl acetate	14.13	0.01 ± 0.002	0.87 ± 0.396	1.34 ± 0.564
		(*Z*)-3-hexenyl butyrate	17.44	0.011 ± 0.002	0.22 ± 0.181	0.19 ± 0.142
		(*E*)-2-hexenyl butyrate	17.8	0.004 ± 0.001	0.007 ± 0.002	0.006 ± 0.001
First night	Aldehyde	(*E*)-2-hexenal	10.87	0.103 ± 0.013	24.6 ± 7.844	22.5 ± 5.312
	Alcohol	(*Z*)-3-hexenol	15.38	0.032 ± 0.006	9.6 ± 2.028	8.2 ± 3.734
		(*E*)-2-hexenol	15.97	0.296 ± 0.023	4.12 ± 0.955	2.89 ± 0.855
	Hexenylester	(*Z*)-3-hexenyl acetate	13.66	0.165 ± 0.028	10.7 ± 3.621	11.53 ± 4.291
		(*E*)-2-hexenyl acetate	14.13	0.009 ± 0.001	1.14 ± 0.371	1.15 ± 0.306
		(*Z*)-3-hexenyl butyrate	17.44	0.007 ± 0.001	0.022 ± 0.008	0.04 ± 0.022
		(*E*)-2-hexenyl butyrate	17.8	0.002 ± 0	0.006 ± 0.002	0.007 ± 0.003
Second night	Aldehyde	(*E*)-2-hexenal	10.87	0.055 ± 0.009	4.7 ± 1.877	9.5 ± 4.009
	Alcohol	(*Z*)-3-hexenol	15.38	0.034 ± 0.018	4.0 ± 1.225	2.94 ± 0.522
		(*E*)-2-hexenol	15.97	0.177 ± 0.021	0.99 ± 0.427	1.47 ± 0.554
	Hexenylester	(*Z*)-3-hexenyl acetate	13.66	0.089 ± 0.024	4.8 ± 2.114	9.4 ± 4.708
		(*E*)-2-hexenyl acetate	14.13	0.01 ± 0.002	0.74 ± 0.505	1.77 ± 0.972
		(*Z*)-3-hexenyl butyrate	17.44	bld.	0.032 ± 0.019	0.039 ± 0.013
		(*E*)-2-hexenyl butyrate	17.8	bld.	0.005 ± 0.003	0.007 ± 0.002

Mean (±SEM; n = 5) release of GLVs in *D. wrightii* plants in nature. A single not yet fully developed leaf of each plant was mechanically wounded and treated with water (w + w) or *M. sexta* OS (w + OS) during the day (**A**, 1:30–3:30 pm) and during night (**B**, first night, **C**, second night, 0–2 am). Volatiles are listed by chemical classes and in order of their retention time; bld.: below the limit of detection.

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acetate specific glomeruli (and thereby OSNs) we also tested these two compounds separately (***Table 3*** gives composition of each GLV-mixture). Experiments were done in a paired design (***Figure 7A***) to minimize the volatile 'noise' caused by, for example, different numbers of flowers, different grades of damage or different plant ages.

When plants were augmented with isomerically pure mixtures that consisted of either (*Z*)-3 or (*E*)-2-GLVs (aldehyde, alcohol, hexenyl acetate and hexenyl butyrate, ***Table 3***) *Manduca* moths laid significantly more eggs on the (*Z*)-3 than on the (*E*)-2-scented side of the plant (mean ± SEM number of eggs oviposited per plant side: (*Z*)-3-isomers 1.0 ± 0.2, (*E*)-2-isomers 0.5 ± 0.1, ***Figure 7B***). When we compared two GLV mixes that contained all tested (*Z*)-3 and (*E*)-2-GLVs in a balanced isomeric ratio (1:1) or in a high (*Z*)-3 vs (*E*)-2-ratio (9:1), significantly more eggs were oviposited on the sides of the plants that were scented with the higher (9:1) (*Z*)-3/(*E*)-2-ratio (9:1-ratio 1.8 ± 0.2; 1:1-ratio 0.6 ± 0.2; ***Figure 7B***). Finally, when we compared the two different hexenyl acetates, on average one additional egg per plant was oviposited on sides scented with the (*Z*)-3-isomer ((*Z*)-3-hexenyl acetate 1.8 ± 0.3, (*E*)-2-hexenyl acetate 0.9 ± 0.1; ***Figure 7B***).

Discussion

Here we demonstrate that the (*Z*)-3/(*E*)-2-ratio of the GLV bouquet emitted from *D. wrightii* plants differs depending on the presence or absence of *M. sexta* larval oral secretions at sites of simulated feeding-damage. As OS-specific changes in the (*Z*)-3/(*E*)-2-ratio were detectable during one of the two nights in field experiments, this volatile signal may be encountered by ovipositing *Manduca* females

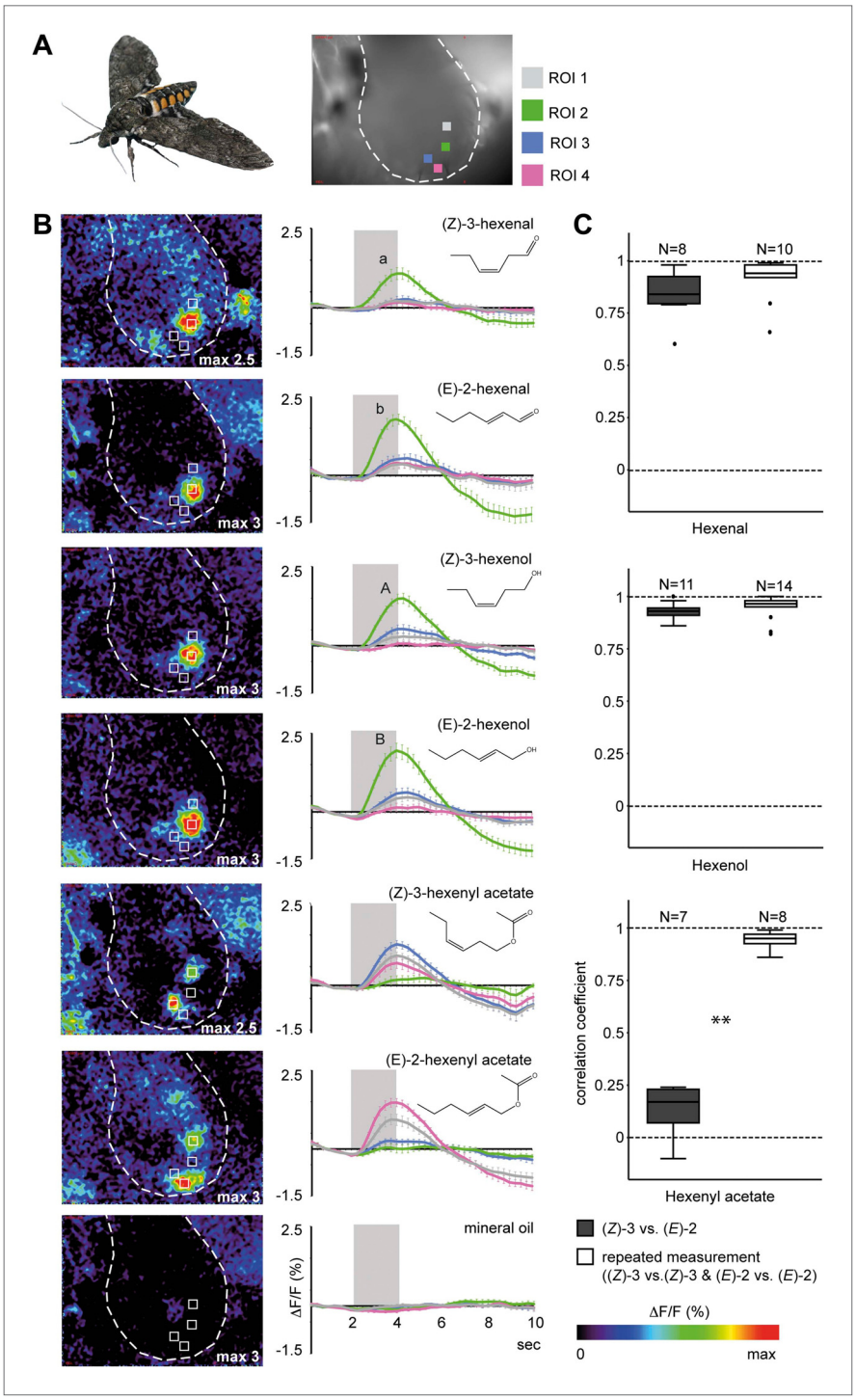

Figure 4. Calcium activity patterns of the (*Z*)-3- and (*E*)-2-isomers in the *M. sexta* antennal lobe (AL). (**A**) View onto the AL (marked by outline) of a *Manduca sexta* female after bath application with the calcium-sensitive dye calcium-green-AM. Stimulations with the six tested GLVs resulted in the activation of four regions in the AL most probably corresponding to single glomeruli (four ROIs, regions of interest). (**B**) Representative false color-coded

Figure 4. Continued on next page

Figure 4. Continued

images show calcium responses in the AL after odor stimulation. Images are individually scaled to the strongest activation (given by the max value in each image). Time traces show activity of ROI 1, 2, 3 and 4 (n = 10) in response to odor stimulation (2 s; grey bar). Error bars represent standard errors of means. For hexenal and hexenol, stimulations with the (*E*)-2-isomers activated ROI 2 significantly stronger than did stimulations with the (*Z*)-3-isomers (Wilcoxon signed-rank test: hexenal: $p<0.01$, hexenol: $p<0.05$). ΔF: change in fluorescence; F: background fluorescence. For raw data, see F4B_AllmannSpaethe2012_timetracesGlvs.xlsx (Dryad: ***Allmann et al., 2012***). (**C**) Comparison of response pattern similarity for repeated stimulations of one structural isomer ((*Z*)-3 vs (*Z*)-3 Or (*E*)-2 vs (*E*)-2, white boxes) and for both structural isomers ((*E*)-2 vs (*Z*)-3, grey boxes); sample size is given above the boxes (Mann–Whitney U test: hexenal: $p>0.05$; hexenol: $p>0.05$, hexenyl acetate: $p<0.001$). For raw data, see F4C_AllmannSpaethe2012_correlationcoefficientsGlvs.xlsx (Dryad: ***Allmann et al., 2012***).

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searching for host plants. Functional imaging experiments revealed that *M. sexta* females detect (*Z*)-3- and (*E*)-2-hexenyl acetate with distinct OSN populations leading to discrete activation patterns in the AL. In field experiments *Manduca* females laid fewer eggs on plants scented with GLV mixtures with increased proportions of (*E*)-2-GLVs.

Our initial laboratory studies indicated that OS-induced changes in the GLV-profile of *Datura wrightii* plants are detectable during day and night, but they also revealed that light plays a role for the magnitude of this change in the signal. It has been shown that darkening can cause a temporary burst of GLVs in plants (***Graus et al., 2004***; ***Brilli et al., 2011***). Furthermore, in *Nicotiana attenuata* the lipoxygenase Na*LOX2*, which specifically provides oxygenated fatty acids for the GLV-pathway, has its highest transcript levels during the night (***Allmann and Baldwin, 2010***), and, while this might explain the overall increase in GLVs with decreasing light intensities, it does not explain the specific increase in (*E*)-2-GLVs in w + w treated plants (***Figure 3*** and ***Table 1***, night). (3*Z*):(2*E*)-enal isomerase activity has been found in crude extracts of some plants (alfalfa and soybean; ***Takamura and Gardner, 1996***; ***Noordermeer et al., 1999***), but not in *N. attenuata* (***Allmann and Baldwin, 2010***), and it needs to be determined whether *Datura* plants possess such an enzyme with a nocturnal peak activity. Circadian rhythm is also known to affect volatile emission (***Goodspeed et al., 2012***) and might therefore be another factor involved in the variation in GLV emission found between the light and dark period.

Most research on herbivore induced plant volatiles has been done in laboratory studies under controlled conditions (***Hunter, 2002***; ***Kigathi et al., 2009***). While these studies provide useful information about the influence of single stress factors, they often fail to include biotic and abiotic stresses that influence volatile production under natural conditions (***Kigathi et al., 2009***). To evaluate the importance of these stresses, we repeated our trapping experiments in the field using native populations of *D. wrightii* plants.

Night-GLV emissions were measured at two different dates; while (*Z*)-3/(*E*)-2-ratios were the same in w + w and w + OS treated plants during the first experimental night, shortly after a new moon, they differed significantly during the full moon, the second experimental night. Although quantitative differences in light intensities between the two experimental nights were not detectable with the instruments available on site, they were obvious to the human eye. The releases of several volatile compounds are known to exhibit diurnal photoperiodicity in their quantitative but also qualitative emission patterns (***Loughrin et al., 1994***; ***Turlings et al., 1995***). In cotton, acyclic terpenes like β-farnesene and β-ocimene were emitted in a diurnal fashion, while GLVs and few terpenes did not show such a clear diurnal pattern (***Loughrin et al., 1994***). Diurnal-rhythm-dependent emission has also been observed in *N. tabacum* after feeding by larvae of *Heliothis virescens*, *M. sexta* and *Helicoverpa zea,* as these plants released larger amounts of (*E*)-2-hexenal during the night and emitted other GLVs exclusively in the dark period (***De Moraes et al., 2001***). Experiments with lima beans revealed that leaves damaged during the scotophase responded with an almost immediate nocturnal emission of (*Z*)-3-hexenyl acetate, while the main emission of β-ocimene was delayed and peaked during the photophase (***Arimura et al., 2008***). These studies affirm that light plays an important regulatory role in volatile emissions. Due to our sample size, it remains to be shown by further experiments whether moonlight is sufficient to regulate volatile emissions.

The herbivore-induced volatile blend comprises several groups of compounds such as GLVs, terpenoids and/or aromatics, all of which have been shown to mediate plant-insect interactions (***Mumm and Dicke, 2010***). GLVs, which were investigated in the present study, seem to play an important role in

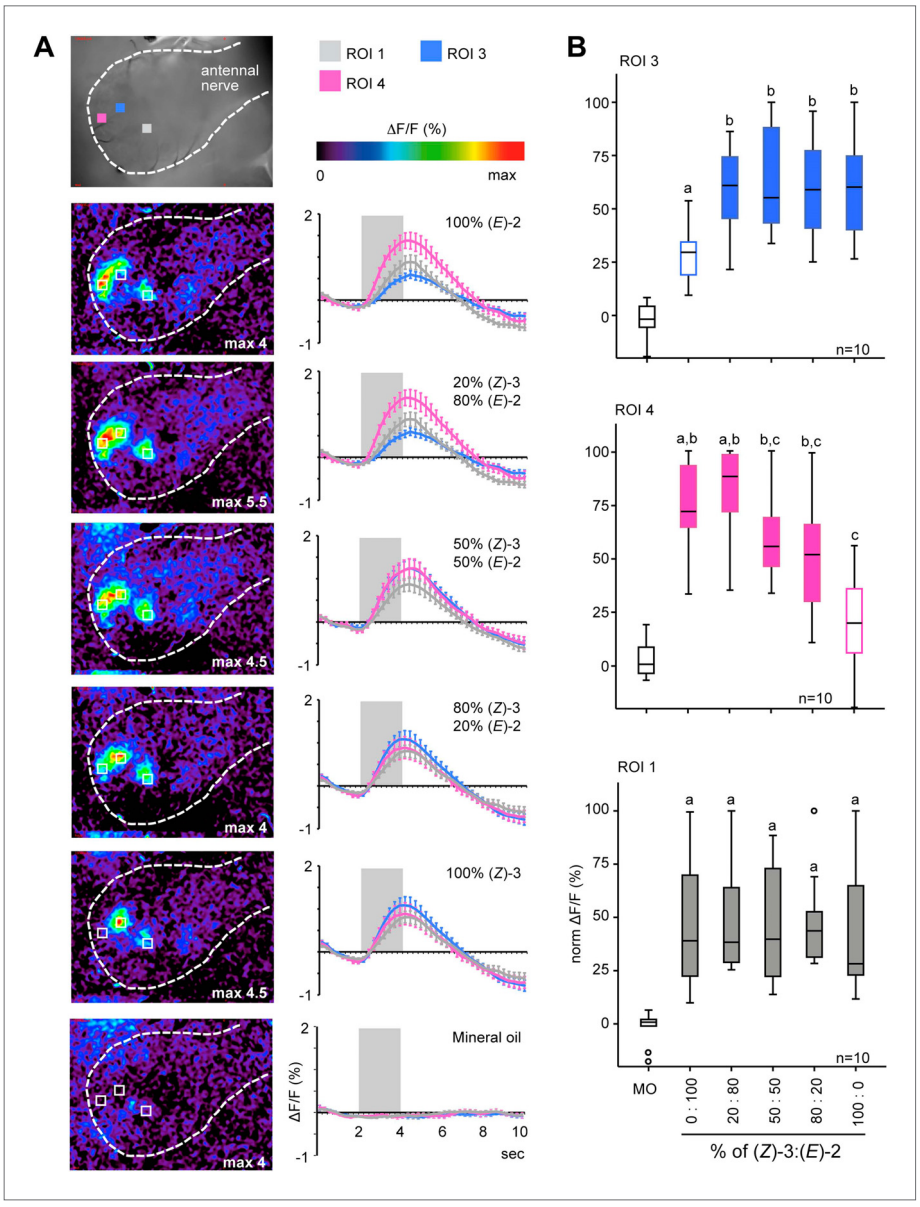

Figure 5. Female antennal lobe (AL) shows isomer-specific calcium responses to (*Z*)-3- and (*E*)-2-hexenyl acetate. (**A**) Representative false color-coded images show calcium responses in the AL after odor stimulation with isomeric mixtures of a total dose of 250 ng. Images are individually scaled to the strongest activation (given by the max value in each image). Time traces show activity of ROI 1, 3 and 4 (n = 10) in response to odor stimulation (2 s; grey bar). Error bars represent standard error of mean. For raw data, see F5A_AllmannSpaethe2012_timetraceshexenylacetate.xlsx (Dryad: ***Allmann et al., 2012***). (**B**) Change in fluorescence in ROI 1, 3 and 4 to the pure structural isomers and their mixtures, normalized to the highest activation in every animal. Filled boxes represent responses significantly different from the mineral oil (MO) control; different letters denote significantly different calcium responses (Kruskal–Wallis and Dunn's multiple comparison test). For raw data, see F5BCE_AllmannSpaethe2012_imaginghexenylacetate.xlsx (Dryad: ***Allmann et al., 2012***).

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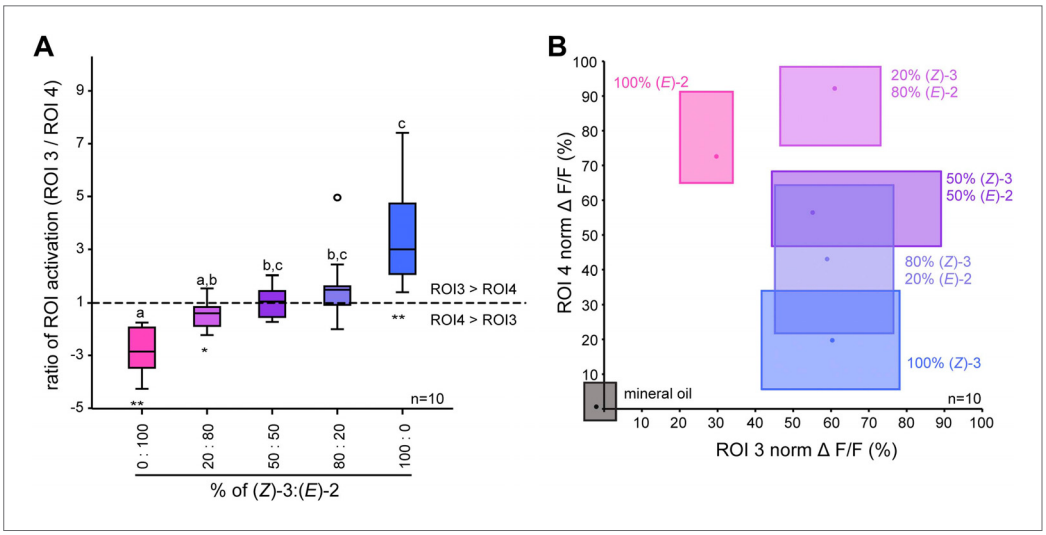

Figure 6. Isomer-specific regions show different response characteristics. (**A**) Both isomer-specific regions ROI 3 and ROI 4 are shown as ratios of ROI activation (ROI 3/ROI 4; for ROI 4 > ROI 3: −1/ratio) at stimulations with 250 ng. Asterisks indicate significant differences from 1, the ratio at which activation would be equal for ROI 3 and 4 (Wilcoxon signed-rank test, 100/0, 0/100: $p<0.01$, 20/80: $p<0.05$). Structural isomers and their mixtures were tested with Kruskal–Wallis and Dunn's multiple comparison test, different letters denote significantly different ratios. (**B**) Calcium signals of ROI 3 (x-axis) and ROI 4 (y-axis) (% norm ΔF/F, separated by axes) in response to odor stimulation (colored boxes) and the solvent mineral oil (grey box). Points denote the median values, box outlines mark the 25% and 75% percentiles. For raw data, see F5BCE_AllmannSpaethe2012_imaginghexenylacetate.xlsx (Dryad: ***Allmann et al., 2012***).

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volatile 'communication' as almost every green plant releases them upon various stress conditions. Furthermore, GLVs are released instantly from plant tissue upon damage, independent of the time of day (***Turlings et al., 1995***; ***D'Auria et al., 2007***), while terpenoids are released with a delay (***Kant et al., 2004***) and several terpenoids not at all during the night as they are linked to photosynthesis (***Arimura et al., 2008***). This makes GLVs an important cue for ovipositing *Manduca* moths as they are active during sunset and night (***Theobald et al., 2010***) and thus need to rely on signals that are released during the scotophase.

The use of volatile blends for host location by insects depends heavily on the ability to detect and process olfactory signals. The insect's olfactory system is highly sophisticated and enables detection of odors at very low concentrations (***Hansson et al., 1999***; ***Tanaka et al., 2009***). However, in a redolent world, insects must distinguish host odors from a high background noise. Plant volatiles are detected by OSNs and these can be tuned to highly specific (***Bruce and Pickett, 2011***) or to ubiquitous host plant compounds (***Hansson et al., 1999***; ***Bruce et al., 2005***). We found that stimulations with hexenal- and hexenol-structural isomers led to activation of a distinct region in the AL (ROI 2, ***Figure 4A,B***). However, calcium signals evoked by (*E*)-2-GLVs were significantly stronger compared with those evoked by (*Z*)-3-GLVs (***Figure 4B***). This difference in activation intensity is likely a result from different binding affinities of the structural isomers to the olfactory receptor expressed by OSNs targeting the activated glomerulus (***Hallem et al., 2004***; ***Hooper et al., 2009***).

Of all tested compounds, only the (*Z*)-3- and (*E*)-2-isomers of hexenyl acetate activated two different discrete regions in the AL of *M. sexta* females (***Figures 4B,C and 5A,B***) strongly suggesting different isomer-specific OSN populations on the insect antenna. This leads to the proposition that for *M. sexta* females, changes in the volatile emission of (*Z*)-3- and (*E*)-2-GLVs might primarily be detected via hexenyl acetate. Given that all other tested GLVs activated only ROI 2, the investment in isomer-specific receptors and consequently glomeruli to detect and process ubiquitous GLV compounds such as (*Z*)-3- and (*E*)-2-hexenyl acetate indicates the importance of the information content transferred by these compounds and their respective ratios. Specific responses to different types of green leaf volatiles have been reported both at physiological (***Hansson et al., 1999***; ***Røstelien et al., 2005***) and behavioral levels (***Reinecke et al., 2005***).

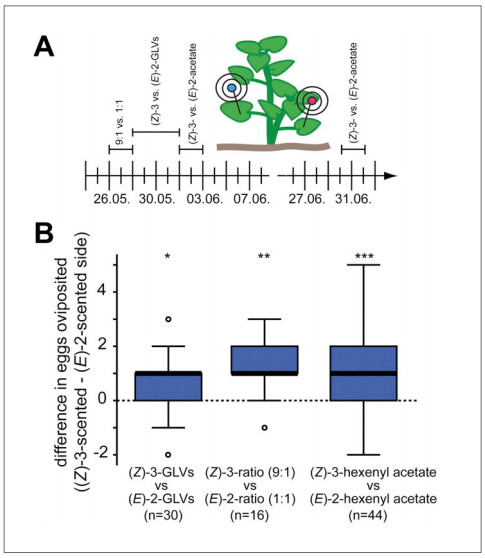

Figure 7. *Manduca* moths laid more eggs on the (*Z*)-3- than on the (*E*)-2-scented side of the plant. (**A**) The effect of different GLV-mixes on the oviposition behavior of female *Manduca* moths was tested during the 2010 field season on native *Datura wrightii* plants. On each experimental day, two different mixes were tested in a paired design. Mixes used on different experimental days are plotted above the timeline. The detailed composition of each mixture is described in ***Table 3***. (**B**) Difference in number of eggs oviposited per plant. Higher oviposition rates were observed for the (*Z*)-3-scented side of the *D. wrightii* plants. Treatment pairs with no oviposited eggs were excluded prior to the statistical analysis (Wilcoxon signed-rank test). The median is represented as a line in each box, box outlines mark the 25% and 75% percentiles; outliers are depicted as circles (if value > 1.5× the interquartile range). For raw data, see F6B_AllmannSpaethe2012_oviposition.xlsx (Dryad: ***Allmann et al., 2012***).
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For hexenyl acetate, AL activation patterns elicited by stimulations with mixtures of both structural isomers were purely additive suggesting no mixture interaction at the OSN and AL input levels, which is consistent with other studies (***Deisig et al., 2006***; ***Carlsson et al., 2007***; ***Silbering and Galizia, 2007***; ***Kuebler et al., 2012***). When comparing the ratio of ROI activation, we did not find any difference between the mixtures (***Figure 6A***). This result is not surprising when taking the different response characteristics of ROI 3 and 4 into account. Calcium activity of ROI 4 in response to mixtures with increasing percentages of the (*E*)-2-isomer were dose-dependent, whereas the activation of ROI 3 to the same mixtures resembled more an 'on–off' mechanism and was thus solely dependent on the presence of the (*Z*)-3-isomer, leading to a constant bias towards (*Z*)-3-hexenyl acetate (***Figures 5B and 6B***). We can, however, not neglect the possibility that the logarithmic, dose-dependent phase in the neural dynamics of the neurons innervating ROI 3 lies at a concentration range below what was tested here.

The different response characteristics of ROI 3 and 4 might mirror the relevance of the odors for *M. sexta* females. (*Z*)-3-hexenyl acetate is a rather ubiquitously occurring plant volatile, which is released in large amounts after damage irrespective of its origin (***Arimura et al., 2008***; ***Mumm and Dicke, 2010***). Electrophysiological experiments revealed that this compound elicited many responses in OSNs on the female *M. sexta* antenna: 60% of the tested sensilla (***Spaethe et al., 2013***) as well as 21 of 34 cells in the female AL (***Kuebler et al., 2011***) responded to this compound. (*E*)-2-hexenyl acetate, in contrast, has rarely been reported in insect-plant interactions (***Whitman and Eller, 1990***; ***Quiroz and Niemeyer, 1998***; ***Williams et al., 2010***), aside from its release among other GLVs after larval feeding of *M. sexta* on *N. attenuata* (***Allmann and Baldwin, 2010***) as an indication of actual larval damage. Thus, the presence of each structural isomer contains specific information but at different levels of resolution and in different contexts. In the case of (*Z*)-3-hexenyl acetate, the detected signal might also be relevant in long-range host location and host choice. Information about (*E*)-2-hexenyl acetate gained by ROI 4 in a dose-dependent fashion should, on the other hand, be most valuable at a short distance to the plant, possibly to locate the best spot for oviposition depending on the actual amounts emitted by different plant sites or to choose among neighboring plants with different levels of (conspecific) herbivory.

Numerous studies suggest that the ratio of plant volatiles is an important component of the olfactory signal (***Visser and Avé, 1978***; ***Bruce et al., 2009***; ***Cha et al., 2011***). ***Visser and Avé (1978)*** found several GLVs playing important roles in host recognition of *Leptinotarsa decemlineata*. Augmenting the volatile emission of a potato host plant with the single GLV components (*Z*)-3- or (*E*)-2-hexenol, (*E*)-2-hexanal or 1-hexanol resulted in a disruption of the orientation of *L. decemlineata* to the potato plant. Further studies found neurons specifically responding to these GLVs both at the periphery and in the AL of *L. decemlineata* (***De Jong and Visser, 1988a***, ***1988b***). In the case of the oriental fruit moth, *Grapholita molesta,* the ratio of a minor compound to the remaining components of a plant-derived

Table 3. GLV-mixtures used for oviposition assay in the field

	Compounds (common names)	***(Z)-3/(E)-2*-mix 1:1; ≈ w + OS (µg/µl lanolin)**	***(Z)-3/(E)-2*-mix 9:1; ≈ w + w (µg/µl lanolin)**
(Z)-3-GLVs	*(Z)*-3-hexenal (50% in triacetin)	5.0	9.0
	(Z)-3-hexenol	5.0	9.0
	(Z)-3-hexenyl acetate	0.05	0.09
	(Z)-3-hexenyl butyrate	0.05	0.09
(E)-2-GLVs	*(E)*-2-hexenal	5.0	1.0
	(E)-2-hexenol	5.0	1.0
	(E)-2-hexenyl acetate	0.05	0.01
	(E)-2-hexenyl butyrate	0.05	0.01
	Triacetin per 10 mL mix (derived from *(Z)*-3-hexenal), µl	51.25	92.2
	Triacetin added per 10 mL mix, µl	40.95	0
	Total amount of triacetin per 10 mL mix, µl	92.2	92.2
	Compounds (common names)	**Only *(Z)*-3-mix (µg/µl lanolin)**	**Only *(E)*-2-mix (µg/µl lanolin)**
(Z)-3-GLVs	*(Z)*-3-hexenal (50% in triacetin)	10.0	0.0
	(Z)-3-hexenol	10.0	0.0
	(Z)-3-hexenyl acetate	0.1	0.0
	(Z)-3-hexenyl butyrate	0.1	0.0
(E)-2-GLVs	*(E)*-2-hexenal	0.0	10.0
	(E)-2-hexenol	0.0	10.0
	(E)-2-hexenyl acetate	0.0	0.1
	(E)-2-hexenyl butyrate	0.0	0.1
	Triacetin per 10 ml mix (derived from *(Z)*-3-hexenal), µl	102.5	0
	Triacetin added per 10 ml mix, µl	0	102.5
	Total amount of triacetin per 10 ml mix, µl	102.5	102.5
	Compounds (common names)	**(Z)-3-hexenyl acetate (µg/µl lanolin)**	**(E)-2-hexenyl acetate (µg/µl lanolin)**
	(Z)-3-hexenyl acetate	5.0	0.0
	(E)-2-hexenyl acetate	0.0	5.0

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synthetic mixture determined behavioral acceptance of this mixture, which could be associated with the response of two glomeruli in the female AL (***Piñero et al., 2008***; ***Najar-Rodriguez et al., 2010***). We found that ovipositing *Manduca* moths distinguished between different (*Z*)-3/(*E*)-2-ratios and that they used these volatile cues to choose oviposition sites associated with less feeding competition and predation. However, independent of whether the mixtures tested were rather complex in their composition (9:1 vs 1:1 ratios), or less complex (only (*Z*)-3-GLVs vs only (*E*)-2-GLVs), or consisted of only a single compound ((*Z*)-3-hexenyl acetate vs (*E*)-2-hexenyl acetate) ovipositing *Manduca* moths continuously made a choice and always preferred the side of the plant that smelled more of (*Z*)-3-GLVs, or less of (*E*)-2-GLVs (***Figure 7B***). From our results we cannot conclude whether a complex GLV bouquet of different ratios provides more reliable information than do single compounds, but our results demonstrate that by adding a single component to the volatile bouquet of native *D. wrightii* plants one can alter the choice of ovipositing *Manduca* moths.

How did this behavior evolve? It has been shown that *M. sexta* moths learn to feed from flowers of non-hosts due to olfactory conditioning (***Riffell et al., 2008***, ***2013***). Experience could as well shape

female oviposition choice as it has been shown in other moth species (**Rietdorf and Steidle, 2002**; **Olsson et al., 2006**). During oviposition *M. sexta* females might encounter the herbivory-specific signal, but can never experience the reward of oviposition success associated with it. A *M. sexta* larva feeding on plants, however, is continuously surrounded by GLVs emitted from wounded plants and more importantly encounters almost continuously a low (*Z*)-3/(*E*)-2-ratio caused by its own oral secretions. The retention of odor memory learned at the larval stage onto the adult stage has been shown to occur in *M. sexta* (**Blackiston et al., 2008**). However, in such a case you would rather expect a preference for OS-elicited bouquets, as the larva grew up in these. More experiments are needed to solve whether experience and learning are involved in the avoidance of herbivory-specific (*Z*)-3/(*E*)-2-ratios.

Almost every green plant releases volatiles in highly variable amounts and compositions. This makes it a challenge for host searching insects to simultaneously extract useful information while flying through the odor plumes from multiple sources. Our results show that the AL, the first odor processing center of the insect brain, has the capacity to resolve the composition of GLV blends as emitted by highly relevant host plants. Correspondingly, gravid females make an informed choice. They prefer oviposition sites with reduced predation and competition risks for their offspring, as indicated by the plant volatile bouquet. Future work will reveal whether increasing amounts of (*E*)-2-GLVs or rather changes in the (*Z*)-3/(*E*)-2-ratio at the background of other host odors provide crucial information for female *Manduca* moths.

Material and methods

Plant material and growing conditions

Datura wrightii seeds were initially purchased from B & T World Seeds (Paguignan, France) and subsequently harvested from plants propagated in the glasshouse. Plants were grown in 2 l pots in the glasshouse (23–25°C, 50–70% humidity, 16 hr light supplemented by Philips Sun-T Agro 400 W Na-vapor bulbs, 350–500 µmol/m^2/s^1 photosynthetic photon flux at plant level) and used for experiments 35–0 days after sowing.

For field experiments we used native populations of similar sized *D. wrightii* plants, which were located close to the Lytle Preserve research station. Wild plants at the field site and plants grown from purchased seeds showed high morphological similarity.

Plant treatments

For all treatments, plants were wounded with a fabric pattern wheel to punch three rows of holes on each side of the midrib. Wounded leaves were immediately treated with 20 µl of deionized water (w + w) or with 1:3 (vol/vol) diluted *M. sexta* oral secretions (w + OS), which were pipetted directly onto the wounded leaf and gently dispersed across the surface. The OS was collected from third to fifth instar caterpillars which were fed on *D. wrightii* plants, and OS was stored under Argon at −20°C until usage.

Volatile collection

Volatile collections were performed in a growth chamber (temperature 23–25°C, humidity 50–60%) on shelves equipped with diode arrays of white (approximately 420–690 nm), red (630–690 nm) and UV (380–420 nm). Diode arrays were programmed to simulate daylight, twilight and night conditions accordingly regarding both light intensity and spectral composition (16:8 hr light/dark cycle, **Figure 2**).

D. wrightii plants were placed in the chamber two days prior the experiment to acclimatize. On the experimental day 1 single leaf per plant was enclosed immediately after treatment between two 50-mL food-quality plastic containers (Huhtamaki, Bad Bertrich, Germany) secured with miniature claw-style hair clips. Ambient air was pulled through the collection chamber and a glass tube (ARS, Inc., Gainsville, FL; www.ars-fla.com) packed with glass wool and 20 mg of Super Q (Alltech, Düsseldorf, Germany; www.alltech.com). Airflow was created by a vacuum pump (model DAAV114-GB; Gast Mfg, Benton Harbour, MI; www.gastmfg.com) as described by **Halitschke et al. (2000)**. For each time point and each treatment we trapped volatiles from five replicate plants. Directly after volatile sampling, we determined the fresh mass (FM) of each trapped leaf for further calculations.

In the field, we selected eight plants of approximately similar size for each measurement in a 10-m radius. For each plant, we estimated the total leaf damage and we counted the number of flowers. To account for differences in volatile emissions caused by different degrees of leaf damage we selected three equal sized leaves of each plant and randomly assigned each leaf to one of the treatments (control, w + w or w + OS). Each leaf was photographed to calculate the leaf area. We subsequently subtracted the amounts of volatiles emitted from untreated control leaves from those emitted from treated leaves of the same plant. We used a Li-COR Li-250A light meter with a Li-190SA quantum sensor (http://www.licor.com) to measure the photosynthetic active radiation during the different trapping periods. Weather data during the volatile collection were obtained from weather station KUTSTGEO6 located in St. George, UT (www.wunderground.com). The first two trappings were performed on the 3 and 4 of June, soon after new moon. During the day volatiles were sampled at an average light intensity of 1450 µmol/s/m^2. During the night samplings, the light intensity was below the detection limit. The second trapping was performed in the night from the 14 to the 15 of June. Although it was a bright night (full moon) the average light intensity remained below the detection limit. On the experimental day, we enclosed single leaves directly after elicitation in polystyrene chambers fitted with holes at both ends. Air was pulled through the chamber and subsequently through a single-use charcoal trap (Orbo M32; Sigma-Aldrich, Seelze, Germany) as described in ***Kessler and Baldwin (2001)***. Charcoal traps were equipped with MnO_2-coated copper gauze as ozone scrubbers (OBE Corporation, Fredericksburg, TX) to prevent oxidation of volatiles.

In all experiments, volatiles were trapped for 2 hr immediately after elicitation.

Both charcoal and SuperQ traps were eluted with 250 µl dichloromethane (DCM) into a GC vial after spiking each SuperQ trap with 320 ng and each charcoal trap with 240 ng tetralin (Sigma-Aldrich, Seelze, Germany) as an internal standard.

Volatile analysis and quantification

Samples were analyzed on an Agilent 7890A gas chromatograph (Agilent Technologies, CA) with the injection port kept at 230°C, operated in split-less mode and connected to an Agilent 5975C mass spectrometer. One microliter of each sample was injected on a polar column (Innowax; 30 m, 0.25 mm ID, 0.25-µm film thickness; J&W Scientific, Folsom, CA) operated under a constant He flow of 1.1 ml/min. The GC oven was programmed to hold 40°C for 5 min, to increase the temperature at 5°C/min to 130°C, then increasing temperature at 30°C/min to a maximum of 240°C. The maximum temperature was held for 15 min. The transfer line to the MS was kept at 260°C. The MS was operated in electron impact mode (70 eV) with the ion source at 230°C and the quadrupole at 150°C. The detector monitored selected ions (SIM): hexenals: *m/z* 55, 69, 83; hexenols: *m/z* 55, 57, 67, 82; hexenyl acetates: *m/z* 67, 71, 82; tetralin: *m/z* 104, 132.

Retentions times for each GLV were ascertained using standards of (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, (Sigma-Aldrich, Seelze, Germany) and quantifications were done after normalization to the peak of IS tetralin with calibration curves for each compound (33, 10, 5, 1, 0.5, and 0.1 ng; n = 3 replicates) using single ion traces (hexenal *m/z* 83, hexenol and hexenyl actetate *m/z* 82). Emission rates were calculated based on fresh mass or surface area of the sampled leaves.

(*Z*)-3/(*E*)-2-ratios were calculated for each sample dividing the amount of the *(Z)*-3-GLV by the amount of its corresponding *(E)*-2-isomer. For visual simplifications (*Z*)-3/(*E*)-2-ratios <1 were depicted as their negative reciprocal.

Insect rearing

M. sexta females were reared as described in ***Grosse-Wilde et al. (2011)***. Pupae were kept separately in paper bags at 25°C and 70% relative humidity under a 16:8 hr light/dark cycle. Naïve adult females were used in functional imaging experiments 2–4 days *post* emergence.

Preparation and staining of adult females of *Manduca sexta*

Moths were restrained in 15-ml Falcon tubes with the head exposed and fixed with dental wax (Surgident; Heraeus Kulzer, Dormagen, Germany). The head capsule was opened and all tissues covering the antennal lobes were carefully removed. The brain was bathed with Calcium Green-2 AM (30 µmol; Invitrogen, Darmstadt, Germany, http://www.invitrogen.com) containing physiological saline solution (***Christensen and Hildebrand, 1987***) with 6% Pluronic F-127, (Invitrogen) for 90 min at 4°C. After staining the brain was rinsed several times with Ringer's solution to remove remaining dye.

Table 4. Average (±SD) GLV emissions of GLV-mixtures used for the field bioassays (cotton swab, after ***Allmann and Baldwin, 2010***) and of native *Datura wrightii* plants in the field during the first 2 hr after w + w or w + OS treatment; second night (0–2 am)

	Volatile release in µg/30 min			
	Cotton swab (after *Allmann and Baldwin, 2010*)		*D. wrightii* leaf	
Common names	9:1 GLV mix	1:1 GLV mix	w+w	w+OS
(*Z*)-3-hexenol	9.8 ± 13.21	7.1 ± 9.71	0.20 ± 0.17	0.15 ± 0.07
(*Z*)-3-hexenyl acetate	0.18 ± 0.21	0.15 ± 0.18	0.24 ± 0.30	0.47 ± 0.67
(*E*)-2-hexenal	1.3 ± 2.14	4.3 ± 7.20	0.24 ± 0.27	0.48 ± 0.57
(*E*)-2hexenol	1.3 ± 1.84	8 ± 11.39	0.05 ± 0.06	0.07 ± 0.08
(*E*)-2-hexenyl acetate	0.06 ± 0.07	0.16 ± 0.20	0.04 ± 0.07	0.09 ± 0.14

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Optical imaging of the antennal lobes

For imaging we used a Till Photonics imaging system (Martinsried, Germany) equipped with a CCD camera (Sensicam; PCO Imaging) connected to an upright microscope (Olympus BX51WI). Monochromatic excitation light was given at 475 nm (500 nm SP; Xenon arc lamp, Polychrome V) and fluorescence was detected with a LP515 emission filter and transmitted by a DCLP490 dicroic filter. The set-up was controlled by software Tillvision 4.0 (Till Photonics). Images were taken with a water immersion objective (Olympus, 10×/0.30). Four-fold symmetrical binning resulted in image sizes of 344 × 260 pixels with one pixel corresponding to an area of 2.5 × 2.5 µm (10× magnification).

Odorants tested with optical imaging

Odors were chosen based on the results of a previous study (***Allmann and Baldwin, 2010***) and on volatile collections of *D. wrightii* plants performed for this study (***Figure 1***; (*Z*)-3- and (*E*)-2- hexenal, hexenol and hexenyl acetate [Sigma Aldrich, Seelze, Germany]). Odors were diluted in mineral oil and used in doses of 25, 250, and 2500 ng for the comparison of pure structural isomers. Hexenyl acetate was additionally tested as percentage mixtures of its (*Z*)-3- and (*E*)-2-isomers ranging from 0/100%, 20/80%, 50/50%, 80/20% to 100/0% (vol/vol) in doses of 250 and 1250 ng.

Odorant stimulation

6 µl of the odorant mixtures were pipetted prior the experiment on a filter paper (Whatman, http://www.whatman.com/) in glass pipettes using doses of 25, 250, 1250 (isomeric mixtures) and 2500 ng, respectively. The same volume of mineral oil served as a control stimulus. The stimulus loaden pipette and a second empty pipette were inserted in parallel into a glass tube, which delivered a constant flow of clean humidified air (1 l/min) along one antenna. A continuous clean airstream (0.1 l/min) was directed through the empty pipette and switched to the odor-containing pipette

Table 5. Average (*Z*)-3/(*E*)-2-ratios of GLV-mixtures used for the field bioassays (cotton swab, after ***Allmann and Baldwin, 2010***) and of native *Datura wrightii* plants in the field during the first 2 hr after w+w or w+OS treatment; 2nd night (0–2am)

	(*Z*)-3/(*E*)-2-ratio of emitted GLVs			
	Cotton swab (after *Allmann and Baldwin, 2010*)		*D. wrightii* leaf	
Common names	9:1 GLV mix	1:1 GLV mix	w + w	w + OS
Hexenol	8.37	1.07	4.44	2.38
Hexenyl acetate	3.24	0.90	25.94	15.67

Emissions of *D. wrightii* were adjusted from leaf surface (cm^2) to fresh mass (g) scale by the rough estimate of 50 cm^2 = 1 g and represent the emission of two medium sized leaves.

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Table 6. Comparison of trapping capability of adsorbents used in laboratory (SuperQ) and field volatile collections

	Peak area × 10^6 ± SD		
Compound name	**RT**	**Activated charcoal**	**SuperQ**
(*Z*)-3-hexenal	2.40	0.90 ± 0.13	5.4 ± 1.01
(*E*)-2-hexenal	3.57	9.3 ± 3.33	6.9 ± 1.21
(*Z*)-3-hexenol	3.35	10.1 ± 3.07	7.6 ± 1.49
(*E*)-2-hexenol		n.d.	n.d.
(*Z*)-3-hexenyl acetate	12.25	0.56 ± 0.28	0.47 ± 0.22
(*E*)-2-hexenyl acetate	12.68	0.52 ± 0.03	0.59 ± 0.04

Mean (±SD; n = 6) peak areas of GLVs emitted from *N. attenuata* plants in the glass house. Of each plant two equally sized leaves were mechanically wounded. Subsequently, volatiles were collected for 1 hr with traps filled with either SuperQ or activated charcoal. Traps were eluted with 250 µl Dichloromethane and measured on a GC-MS equipped with a BR-5ms column (Bruker, 15 m, 0.25 mm ID, 25 µm).

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(Syntech Stimulus Controller CS-55) during stimulation, thus preventing any change in total flow during the experiment.

Every stimulation experiment lasted for 10 s, recording 2 s pre- and 6 s post-stimulus and 2 s of odor stimulation. Inter-stimulus time of at least 1 min was chosen to reduce adaptation effects. Every odor was presented first in the lower concentration. The sequence of the stimulations was changed from animal to animal. In some females (hexenal n = 10, hexenol n = 14, hexenyl acetate n = 8), the odors were repeatedly measured to test for the reproducibility of the evoked activity patterns within an animal (***Figure 4C***).

Processing of optical imaging data

All stimulation experiments were recorded with 4 Hz resulting in a series of 40 consecutive frames, which were analyzed with custom written software (IDL; ITT Visual Informations Solutions). Data were corrected for background fluorescence, bleaching of the dye and movement during the measurement (***Sachse and Galizia, 2002***, ***2003***). A spatial median filter of 5 pixels was applied to reduce shot noise.

Odor responses represented as change in fluorescence (ΔF/F) at spatially distinct activity spots were analyzed at the spot center in an area of the size of a small to medium-sized glomerulus (60 × 60 µm). Time traces of ΔF/F were smoothed by averaging three successive frames for each activity spot. The maximum ΔF/F value after stimulus onset was averaged with the pre- and postmaximum value. For every animal the odor responses were normalized to the maximal response and were taken into account if they reached ≥30% of the maximal value in this animal in at least one activity spot.

Analysis of activity patterns in the moth antennal lobe

Due to the lack of a glomerular map in the *M. sexta* AL observed activity regions for the tested odors could not be directly compared between animals. Thus, activation patterns for every isomeric pair of hexenal, hexenol and hexenyl acetate were used to calculate correlation coefficients providing a relative measurement of similarity (***Bisch-Knaden et al., 2012***). Repeated stimulations with the same structural isomer and the correlation coefficients thereof were used as control.

To compare activity patterns between the different isomeric mixtures of hexenyl acetate we calculated the difference in activity of both isomer-specific glomeruli resulting from the ratio of activity in the (*Z*)-3-specific and the (*E*)-2-specific glomerulus. For visual simplifications values below 1 (representing cases in which the (*E*)-2-specific glomerulus was more active than the (*Z*)-3-specific glomerulus) were displayed as their negative reciprocal and all values were presented on a scale without the range between '−1' and '1' (***Figure 6A***).

Oviposition assay in the field

Experiments were done between 26 May and 1 July 2010 in southwestern Utah. This area is part of the native habitat of the tobacco and tomato hawkmoths *Manduca sexta* and *M. quinqemaculata*. Eggs of both species were counted for this experiment. We selected between 15 and 17 plants of two native

populations of *D. wrightii* plants, which were located close to the Lytle Preserve research station. All plants were carefully inspected and oviposited *Manduca* eggs were removed prior the experiment. On each experimental day two mixes were tested in a paired design: every evening before sunset (at around 5 pm) cotton swabs were dipped into the GLV-scented lanolin pastes and stuck onto two opposing branches of one *Datura* plant (***Figure 7A***). This paired design was chosen to minimize the effect that different numbers of flowers or different grades of leaf damage might have on the oviposition behavior of the moths. On the next day freshly laid *Manduca* eggs were counted in a defined area on the plant, approx 30 cm around the scented cotton swabs and afterwards removed. Treatment sides were switched every day. Plants with no oviposited eggs (isomers N = 36, ratios N = 15, acetates N = 18) were excluded prior to the statistical analysis. The GLV-scented lanolin pastes were prepared by warming up lanolin and adding different GLV-mixtures to the liquefied lanolin paste shortly before it solidified again. The GLV mixes used are described in ***Table 3***. A comparison of emission rates and (*Z*)-3/(*E*)-2-ratios emitted from cotton swabs and w + OS and w + w treated *D. wrightii* plants is shown in ***Tables 4 and 5***.

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CHAPTER III

Signal intensity affects host selection in *Manduca sexta*

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M. sexta female on *D. wrightii* flower bud. Photo: A. Späthe

ABSTRACT

Host plant choice is of vital importance for herbivorous insects that do not exhibit brood care. Several aspects like palatability, nutritional quality and predation risk have been found to modulate host preference. Consequently, an ability to identify and select among hosts from a distance is important. Olfactory cues are thought to play an important role as cues underlying host orientation and choice. However, the detailed role of olfaction during host choice is not resolved. In this wind tunnel study we offered mated, ready to oviposit female hawkmoths (*Manduca sexta*) two ecologically relevant, attractive, non-flowering host species, which are known to differ in volatile composition and intensity and in degree of attractiveness. By excluding all but olfactory cues, we show that *M. sexta* females use vegetative plant odors to choose their host, clearly preferring the less concentrated bouquet of *Datura wrightii* over the more intense *Nicotiana attenuata* odor. Harmonization of volatile intensity did not equalize but significantly weaken this preference. Furthermore, superimposition of the highly attractive headspaces of both species' abolished attractiveness completely. Our results confirm the role of blend composition in host plant recognition and add stimulus intensity as an important feature affecting olfactory-based host choice.

Keywords: *Manduca sexta*, host plant choice, wind tunnel, host odor

INTRODUCTION

For insects that do not support their offspring after oviposition the choice of host plant plays an essential role to ensure reproductive success. To provide its offspring with an optimal environment the mother needs to take several aspects like palatability, nutritional quality and shelter from enemies into account. The influence of these factors on host plant choice has been studied intensely (Thompson and Pellmyr 1991b, Dicke 2000, Awmack and Leather 2002), but the role of olfaction is still unclear.

Here we investigate host choice of the oligophagous hawkmoth *Manduca sexta.* Adult *M. sexta* forage on nectar from plants of several families, whereas oviposition almost exclusively occurs on solanaceous plants (Yamamoto and Fraenkel 1960, Mechaber and Hildebrand 2000). In the Great basin of Utah *M. sexta* feeds and oviposits on coyote tobacco *Nicotiana attenuata* and jimson weed *Datura wrightii* with the latter being preferred both for nectar feeding (Kessler 2012) and oviposition (Spaethe et al. 2013). Both species, intact and non-flowering, have been shown to emit overlapping but distinct volatile bouquets. Beside qualitative differences, the less

preferred *N. attenuata* is characterised by a 5-fold total volatile emission rate compared to *D. wrightii* (Spaethe et al. 2013).

We ask, (i) whether the preference for *D. wrightii* persists with presentation of exclusively olfactory cues. As the plant volatiles of *D. wrightii* and *N. attenuata* differ both in quality and quantity (Spaethe et al. 2013), we also ask whether (ii) stimulus intensity, i.e. total volatile concentration, contributes to the differential attractiveness and if (iii) the complete host blend, potentially perceived as olfactory host image, is mediating host choice.

MATERIAL & METHODS

Insect & Plants

M. sexta larvae were reared in laboratory as described in Grosse-Wilde et al. (2011). Pupae and adults were kept under an inversed 16 h : 8 h light/dark regime. Naïve females were mated the second night after emergence and tested during the subsequent night. Adults were supplied with sugar solution *ad libitum*.

All plants were grown in a greenhouse as described in Spaethe et al. (2013). Plants used for experiments were not yet flowering. Approximately 10 days before usage plants were transferred into a climate chamber (23-25° C, 60-80% rH) with inversed 16 h : 8 h light regime.

Behavioral experiments

The attractiveness of *D. wrightii* and *N. attenuata* volatiles emissions was assessed in wind tunnel experiments (Figure 1A). Attractiveness of each species was measured by comparing it against a clean air control (Fig 1A - I). Effects of signal intensity were tested within (Fig 1A-III.) and between both species (Fig 1A – IV.).

Wind tunnel assay

The wind tunnel (Plexiglas, LxHxW 2.5x1x1 m) was set to an airflow of 0.4ms^{-1}, 0.5 Lux light, 25°C, and 70% rH. Mated females were tested individually during the first 3 h of scotophase. 1 h before the experiment females were transferred individually into mesh tubes (13 x 15cm). At the start of each experiment a female was placed on a release platform. Females that did not show wing-fanning behavior within 3 min were gently prodded. After take-off females were observed for 4 min noting every contact with the odor sources. Since we were interested in the choice behavior we did only assess females, which contacted the source. Consequently, females that did not show wing-fanning behavior within 5 min or did not contact the odor sources were regarded as non-responders and excluded from the statistical analysis. Females showing

proboscis extension, i.e. nectar-foraging, were also excluded. We evaluated first choices and indices of attraction based on the total number of contacts to the sources: ((contacts A – contacts B) / total contacts) ranging from 1 (absolute preference of source A) to -1 (absolute preference of source B).

Stimulus delivery

All wind tunnel experiments were performed with on-time produced plant headspace delivered at two sources 40 cm apart at the upwind end of the tunnel (Figure 1A). 1 h before the experiment, plants were placed in aluminum-framed glass boxes (LxWxH 40 x 40 x 60 cm) outside the wind tunnel. Aluminum discs excluded soil and roots from the box. Active charcoal-filtered air (1.2 L/min) was introduced from the top, plant headspace was pumped into the wind tunnel (0.8 L/min) and was released below an artificial leaf (approximately 6 x 8 cm) made of light green tissue paper.

To test for stimulus intensity effects we manipulated the headspace presented in the experiment. The total volatile emission of *N. attenuata* was found to be five times higher than the emission of *D. wrightii* (Spaethe et al. 2013). To investigate the importance of headspace concentrations for ovipositing hawkmoths, AC-filtered air was added via a y-connector. Using a ratio of 1:4 (vol/vol) we asked whether (i) a diluted stimulus would be as attractive as the original plant headspace and (ii) if a volatile concentration of a *N. attenuata* headspace adjusted to *D. wrightii* concentrations would reveal concentration effects in host species preferences.

To investigate whether the preference for *D. wrightii* might be linked to a host image composed of the entity of volatiles emitted by the plant we superimposed the headspace of *N. attenuata* and *D. wrightii* by mixing them 1:1 (vol/vol) via a y-connector and tested this mixture against clean air.

RESULTS

Plants versus controls

For the first contact females significantly preferred *D. wrightii* and *N. attenuata* headspace over clean air controls (Fig 1B). The total number of contacts with plant odor sources was significantly higher compared to the control source (table 1) resulting in a significant index of attraction (Fig. 1C). When mixing the two host blends, the preference for the plant headspace was completely abolished (Fig 1 B, C; table 1). Consequently, the index of attraction of the superimposed host blend experiment differed significantly from the indices of the experiments testing the species singly against clean air (Fig 1C, $p<0.0001$, Kruskal-Wallis Test).

Signal attenuation: intraspecific

For both *N. attenuata* and *D. wrightii*, no preference was observed when headspace was presented against diluted headspace from the same species (Fig 1 B, C; table 1).

Table 1. Average number of source contacts (+/- SD).

Experiment	N	average (± SD) number of source contacts		Wilcoxon Signed Rank Test
D. wrightii vs Control	40	*D. wrightii*	2.9 ± 2.9	
		control	0.7 ± 1.1	p < 0.001
N. attenuata vs Control	48	*N. attenuata*	2.1 ± 2.4	
		control	0.8 ± 1.4	p < 0.001
Mixture vs Control	41	*mixture*	3.0 ± 2.4	
		control	3.9 ± 2.9	n.s.
diluted *N. attenuata* vs *N. attenuata*	51	*1/5 N. attenuata*	2.5 ± 2.1	
		N. attenuata	2.4 ± 2.6	n.s
diluted *D. wrightii* vs *D. wrightii*	34	*1/5 D. wrightii*	2.2 ± 2	
		D. wrightii	2.6 ± 2.8	n.s.
N. attenuata vs *D. wrightii*	45	*N. attenuata*	1.1 ± 1.5	
		D. wrightii	2.5 ± 3	p < 0.01
diluted *N. attenuata* vs *D. wrightii*	39	*1/5 N. attenuata*	2.7 ± 2.3	
		D. wrightii	4 ± 3.3	p < 0.05

Signal attenuation: interspecific

Comparing non-manipulated headspace, females showed significantly more first contacts (Fig 1B) and a significantly higher total number of contacts (Fig 1C, table 1) to *D. wrightii*-scented than to *N. attenuata*-scented sources. Diluting the *N. attenuata* headspace 1:4 (vol/vol) with clean air to a volatile concentration similar to that of *D. wrightii* did not abolish the previously observed bias of neither first contacts, nor total number of contacts towards *D. wrightii* (Fig 1C, table 1). Nevertheless, comparing attraction indices between the experiments revealed that diluting the *N. attenuata* blend significantly weakened the preference for *D. wrightii* (Fig 1C, $p<0.05$, Mann-Whitney U Test).

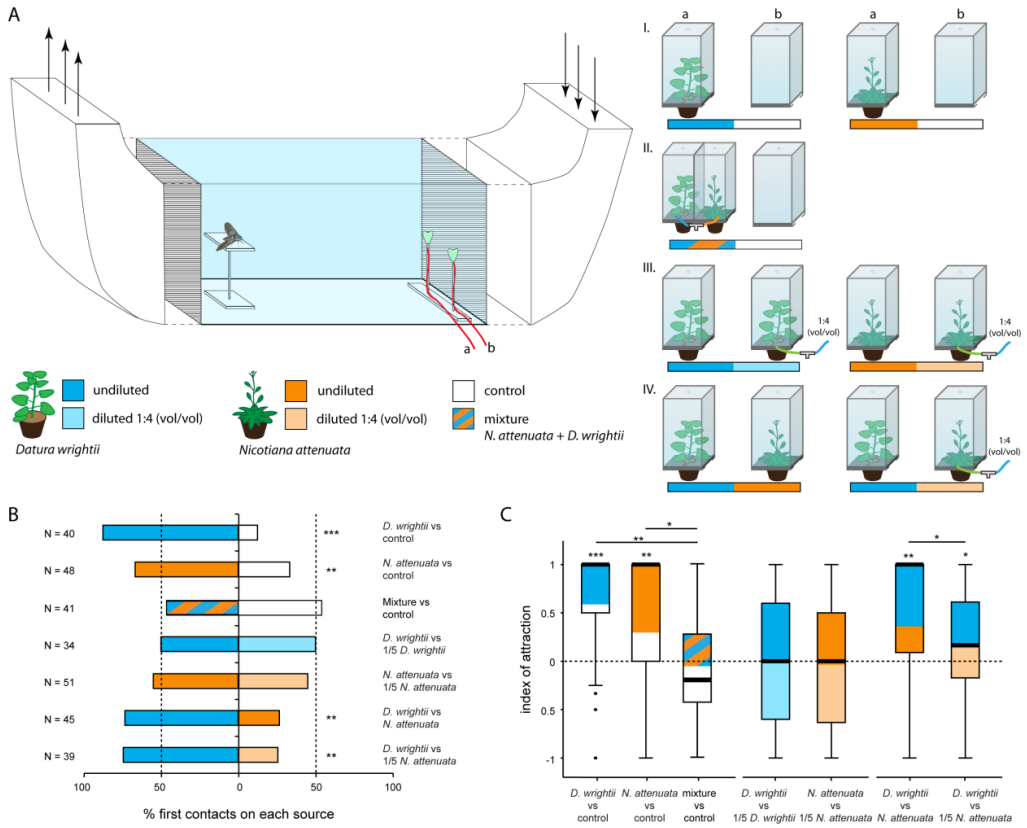

Figure 6.
(A) Choice experiments with gravid *M. sexta* females were performed in a wind tunnel (1 x 1 x 2.5 m). To exclude any but olfactory cues of the host plants, the plants were placed in framed glass boxes outside the wind tunnel. Pumps delivered plant headspace to two sources in the wind tunnel. Identical artificial leafs served as visual stimuli at the two odor outlets. The host plants *D. wrightii* and *N. attenuata* were tested (I) against clean air control, (II) with their headspaces mixed together 1:1 against clean air control, (III) against a conspecific plant whose headspace was diluted 1:4 (vol/vol) with clean air, and (IV) against each other, with *N. attenuata* headspace being unmanipulated or diluted 1:4 with clean air.
(B) Percentage of first choices made in the corresponding experiments. Sample size is given next to each experiment. Asterisks denote significant differences between sources (Chi Square Test, *** p<0.001; ** p<0.01).
(C) Boxplots depict indices of attraction calculated from the number of contacts to each source. Values close to 1 or -1 represent high attraction to one source, 0 means no preference. Black lines delineate the median, color distribution within the box represents the percentage of contacts to each source. Asterisks above the boxes denote indices significantly different from 0 (Wilcoxon signed ranks Test, *** p<0.001; ** p<0.01; * p<0.05). Attraction indices resulting from experiments offering the headspace of both species superimposed or separately against clean air differed significantly (Kruskal-Wallis Test, p<0.0001, and Dunn's posthoc test, ** p<0.01, * p<0.05). Furthermore, attraction indices derived from interspecific choice experiments were significantly different from each other (Mann-Whitney U Test, p<0.05).

DISCUSSION

We show that olfactory stimuli alone are sufficient to elicit differential attraction to host plant species in mated *M. sexta* females. Volatiles from both species are attractive when compared against clean air, and the attractiveness is robust against reduction in stimulus intensity. Simultaneous but spatially separated sources of *N. attenuata* and *D. wrightii* headspace resulted in a strong preference for the latter. This olfaction-mediated host choice corresponds to oviposition preferences previously demonstrated in *Manduca* (Spaethe et al. 2013).

N. attenuata plants emit a 5-fold higher amount of volatiles compared to *D. wrightii* (Spaethe et al. 2013). Although *D. wrightii* headspace was still significantly preferred when reducing volatile

concentration in *N. attenuata* headspace to the "*Datura*" level, the comparison of attraction indices revealed a significant attenuation of the host preference. Thus, stimulus intensity modifies host preference at the species level in a wind tunnel setting.

The observed preference hierarchy, consistent with what has previously been reported (Spaethe et al. 2013), underlines the prominent role of *D. wrightii* as host plant for *M. sexta.* The attractiveness of this species has been associated with the presence of large, night-blooming and intensely scented flowers providing large amounts of nectar to the moth (Adler and Bronstein 2004, Kessler 2012). However, to uncouple host from foraging cues we presented volatiles from non-flowering plants and excluded animals extending their proboscis during flight (less than 6% of tested moths) from the analysis. Similar to what has been shown for flowers (Goyret et al. 2007, Klahre et al. 2011), our results emphasize the important role vegetative plant volatiles play in olfactory remote host recognition and establishment of host choice.

Insects may extract information regarding a volatile-emitting plant through characteristic compounds or compound classes (Fraenkel 1959a, Visser 1986, Bruce et al. 2005b), and species-specific plant volatile blends (Bruce et al. 2005b, Bruce and Pickett 2011). Stimulus concentration has not yet been addressed sufficiently as part of the host information coding mechanism. A comparison of the volatile profiles of *N. attenuata* and *D. wrightii* revealed qualitative as well as quantitative differences: in undamaged plants volatile emission of *N. attenuata* was 5 times higher compared to *D. wrightii* (total emission rate D. wrightii 1.8 +- 0.3 ng/min, *N. attenuata*: 10.5 +- 0.5ng/min, Spaethe et al. 2013) and was composed of numerous typical herbivore-induced plant volatiles (HIPVs), i.e. compounds whose emission gets up-regulated after herbivore attack (Turlings et al. 1995). It has been shown that HIPVs mediate avoidance of damaged plants in ovipositing moths (De Moraes et al. 2001, Kessler and Baldwin 2001). Thus, preference for *D. wrightii* compared to *N. attenuata* may be co-mediated by volatiles associated to herbivore-damaged plants. Consequently, a reduced HIPV-concentration might lead to shifted preference indices as observed in our experiments.

When we presented two blends of identical compositions but different concentrations, moths did not discriminate between full and diluted plant headspaces. While in a turbulent environment distance from a stimulus source has been shown to be mainly coded by stimulus intermittency (Murlis et al. 1992), differences in plant volatile concentration could also have been interpreted by the moth as a function of distance to the plant (Riffell et al. 2008a). However, our results give no indication that a moth would prefer a host-plant blend at a higher concentration. Hence, ovipositing moths seem to predominantly rely on blend composition rather than on concentration.

The picture changed when we diluted the concentration of the complete *N. attenuata* blend to the level of *D. wrightii* and presented both in parallel. While *D. wrightii* usually is strongly preferred over *Nicotiana*, this preference became less accentuated when the *N. attenuata* odor was diluted (Fig. 1C). Thus, stimulus intensity becomes a behaviourally relevant feature of an odor, when different odor sources are available in a repeated choice setting. In a similar way *M. sexta* females preferred oviposition on inbred horsenettle, *Solanum carolinense*, plants, which emit considerably less night-time volatiles compared with outbred plants (Kariyat et al. 2013).

The preference shown for single plant blends against clean air disappeared when *N. attenuata* and *D. wrightii* blends were presented as a mixed stimulus. Clearly, *M. sexta* females could no longer evaluate the olfactory information provided by this combined species mixture, suggesting that the species-specific blend composition contains crucial information. In Colorado beetles the adding of single host volatiles (Visser and Avé 1978a) or a non-host plant (Thiery and Visser 1987) to the blend of a host plant has been shown to neutralize the host's attractiveness. Host recognition in *M. sexta* females is very likely also dependent on ratio specificity. Several studies have reported masking of host plants by repellent or neutral blends (Schroeder and Hilker 2008). However, a reciprocal neutralization of two attractive blends from two naturally attractive host plants as shown in this study has to our knowledge never been reported.

We could show that for female *Manduca sexta* olfactory information emitted by host plants is sufficient to mediate host location and choice. Furthermore, our results highlight the role of blend composition in host plant recognition and add stimulus intensity as another feature involved in this task.

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Odours have a power of persuasion stronger than that of words, appearances, emotions, or will. The persuasive power of an odour cannot be fended off, it enters into us like breath into our lungs, it fills us up, imbues us totally. There is no remedy for it.

Patrick Süskind, The Perfume [3]

GENERAL DISCUSSION

The present thesis elucidates the role of host plant derived volatiles in the choice of egg laying substrates of *M. sexta* applying behavioural, analytical, electrophysiological and Ca^{2+}-imaging methods. Choice of host species is mediated by vegetative plant volatiles: oviposition preferences regarding plant species (chapter I) are consistent with preferences shown with plant headspace volatiles as the only host related cues in wind tunnel experiments (chapter III). *M. sexta* females very likely perform their host choice using a host-specific olfactory 'gestalt', which is mainly characterized by blend composition but can be influenced by volatile signal intensity as well (chapter III). Avoidance of feeding-damaged host plants is commonly associated to predation risk for the offspring on plants that persuasively advertise the presence of the herbivore to the third trophic level. However, for *M. sexta* preference for intact over feeding damaged plants is species-specific (chapter I). Based on discrete chemical profiles, the olfactory system of *M. sexta* is able to discriminate between host species and condition via broadly and narrowly tuned olfactory sensory neurons (OSNs). Some sensory neurons display plant species-specific responses giving rise to overlapping but still distinct activity patterns. In *D. wrightii* feeding *M. sexta* larvae induce an isomerase-mediated shift in the ratio of (*E*)-2- vs. (*Z*)-3-hexenal, as well as the respective alcohols and esters. Ovipositing *M. sexta* use this herbivore-specific signal to avoid already occupied plant areas. Ca^{2+}-imaging revealed that the antennal lobe gets sensory input from configuration-specific OSN populations. Dose and ratio dependent activity patterns reveal that ratios of hexenyl acetate configurational isomers are computed in the antennal lobe (chapter II).

Oviposition preferences: host species

As early as 1960 Yamamoto and Fraenkel presented first oviposition preference experiments with *M. sexta*. Females were provided with a choice between tomato (*Lycopersicon esculentum*) and several solanaceous and non-solanaceous plants. In each trial moths preferred tomato irrespective of the competing species leading to the authors' assumption that at least two

[3] "Es gibt eine Überzeugungskraft des Duftes, die stärker ist als Worte, Augenschein, Gefühl und Wille. Die Überzeugungskraft des Duftes ist nicht abzuwehren, sie geht in uns hinein wie die Atemluft in unsere Lungen, sie erfüllt uns, füllt uns vollkommen aus, es gibt kein Mittel gegen sie."
Patrick Süskind, Das Parfüm, Zürich: Diogenes Verlag, 1994; English version: The perfume, London: Penguin UK, 2006.

stimuli must influence host preference: “One stimulus, which is common to solanaceous plants, apparently triggers oviposition, while the other stimulus, peculiar only to tomato in this particular situation, may serve to orient the moths to the plant, but not to induce oviposition.” We nowadays know that insects identify hosts by typical ratios of host plant compounds, and that adding a single non-host compound or distortion of the ratio may impede host plant location (Bruce et al. 2005a, Bruce and Pickett 2011), but the role of olfaction in the establishment of an oviposition choice among suitable plants is still uncertain.

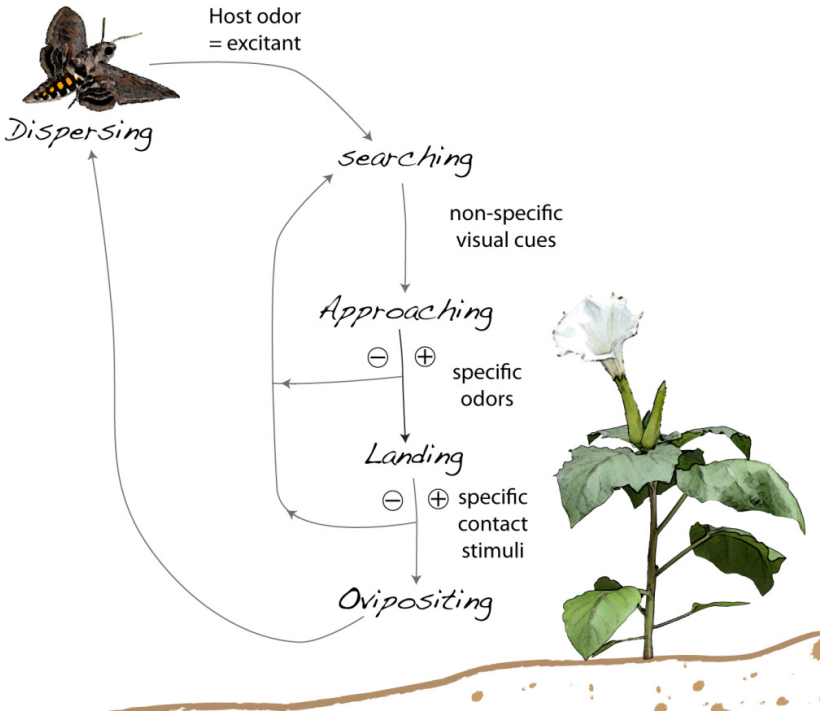

Figure 1. Sensory cues influencing oviposition behaviour of *M. sexta*. Modified after Yamamoto et al. (1969)

Wind tunnel experiments have shown that plant volatiles attract *M. sexta* females from a distance (Mechaber et al. 2002, Fraser et al. 2003, Goyret et al. 2007), but have been dominated by no-choice experiments, a scenario which is rather unlikely in nature. During oviposition females encounter multiple stimuli signifying a potential host plant (Yamamoto et al. 1969) such as visual impressions (Goyret et al. 2007, Goyret et al. 2008) and contact cues (Sparks 1973), which are sensed by gustatory receptors located on tarsi or ovipositor (Honda 1995). Sparks (1973) investigated the effect of contact stimuli using crude plant extracts on artificial plants and showed positive effects of host chemicals, moisture, surface structure and water vapour on *M. sexta* oviposition. Thus, any choice observed in an oviposition experiment may be based on

multimodal perception of plant cues. Based upon observations of ovipositing *M. sexta* moths, Yamamoto et al. (1969) sub-divided oviposition behaviour into sequential steps from searching to oviposition, each influenced by different stimuli (Figure 1). Host odours, they argued, guide moths during the approach to the plant. After landing, however, contact stimuli finalize the sensory path towards oviposition.

The results presented in this thesis concur with previous reports by showing that ovipositing *M. sexta* females discriminate between host and non-host plant species (chapter I) and show a preference hierarchy among host plants (chapter I, III). Going a step further this thesis demonstrates that *M. sexta* females may choose among host plants from a distance using olfactory cues only. In chapter I we report about oviposition experiments where contact to the plant was prevented by hiding plants under gauze tents. The observed oviposition preference coincided with results of experiments allowing full contact. Observation of oviposition behaviour during choice experiments underlined that physical contact to the plant correlated with the number of eggs found on each species. Finally, wind tunnel experiments providing only plant headspaces showed unambiguously that host odours alone suffice to mediate host choice in *M. sexta* females (chapter III). Condensed, these results demonstrate that the oviposition decision is already made from a distance and is based on olfactory cues. Consequently, olfaction constitutes an important, if not essential modality influencing oviposition behaviour. Nevertheless the plant provides visual and contact stimuli as well. So which functions do these cues have? Contact stimuli comprise surface chemical cues like sugars, acids or alkaloids as well as moisture or surface texture. Feeding larvae utilize surface chemicals for host recognition and establishing a feeding preference (del Campo et al. 2001, del Campo and Miles 2003). In adult lepidopteran insects contact cues, especially surface chemicals, have been shown to mediate host acceptance (reviewed by Ramaswamy 1988, Renwick and Chew 1994). Sugars present on the surface of grapes, detected by sensilla on the ovipositor, have been shown to influence oviposition of *L. botrana* moths (Maher et al. 2006). However, when olfactory, visual and contact cues were tested for their influence on host choice, olfaction combined with vision was found to be almost as effective as a full natural stimulus (Tasin et al. 2011). In *M. sexta*, surface moisture was found to affect oviposition (Sparks 1973) suggesting that contact cues might be used to find the right spot on the plant to lay an egg. Contact cues may also serve as a reassuring signal.

Although *M. sexta* is night-active (Madden and Chamberlin 1945, Lingren et al. 1977a) vision is a relevant modality. The hawk moth visual system enables colour vision at starlight level, which is used to locate flowers for nectar foraging (Kelber et al. 2003). *M. sexta* has been shown to

discriminate and learn colours associated with nectar sources (Goyret et al. 2008). Both, visual and olfactory stimuli guide *M. sexta* to nectar-supplying flowers (Raguso and Willis 2002, 2005); and conflicting both stimuli resulted in a preference for the visual cue (Goyret et al. 2007). However, the effect of visual cues was only investigated in association with flowers and consequently, nectar feeding. Whether visual cues from green plants attract gravid *M. sexta* females remains to be explored.

In all host choice experiments presented here *M. sexta* showed a clear preference for *D. wrightii* over *Nicotiana attenuata*. Floral scent and nectar were assumed to mediate attraction to *D. wrightii* plants (Adler and Bronstein 2004, Kessler 2012, Riffell et al. 2013). Both floral volatiles as well as nectar volume have been shown to increase the number of oviposition events (Adler and Bronstein 2004, Reisenman et al. 2010, Kessler 2012). We demonstrated however, that the preference for *D. wrightii* does not depend on flower volatiles. We worked strictly with non-flowering plants. Thus, intense floral odours might strengthen the particular attraction of *M. sexta* to *D. wrightii* plants; host attraction and host choice, however, can be established without them.

Oviposition preference: host condition

Classical field studies have shown that ovipositing *Manduca* moths prefer intact plants over plants that emit herbivore-induced plant volatiles in response to larval feeding damage (Kessler and Baldwin 2001, Kessler and Baldwin 2004). Our laboratory experiments showed consistent results however, the preference between different plant conditions was species-dependent. Only in the case of *N. attenuata*, did *M. sexta* females show a preference for undamaged over feeding-damaged plants. Due to high levels of heterogeneity in oviposition behaviour, this preference was, albeit significant, rather weak suggesting a high flexibility in the evaluation of host plant quality. Overall, the results on host condition concur with the outcome of a recent oviposition study, which was conducted in parallel to our experiments. (Reisenman et al. 2013) applied different treatments to *D. wrightii, D. discolor* and tomato (*Solanum lycopersicum*) plants, disentangling the volatile signal emitted by a herbivore attacked plant. For *D. wrightii* plants, the study failed to show any effect of damage on oviposition choice. In contrast, for tomato plants females responded to larval damage and mechanical damage by showing a preference for intact plants. Taking the results of both studies into account, the particular role of *D. wrightii* among *M. sexta*'s host plants becomes apparent. As other plants *D. wrightii* suffers considerably from herbivory by *M. sexta* larvae (Barron-Gafford et al. 2012). Consequently, *D. wrightii* recruits predators and parasitoids via herbivore-induced volatile emission (Hare and Sun 2011a, chapter II), which results in a larval survival below 0.5 percent in the field (Mira and

Bernays 2002). Contradictory to this elevated predation pressure, *M. sexta* moths did not avoid ovipositing on feeding-damaged plants in the oviposition experiments. Why do ovipositing females evaluate herbivory and the associated predation risk differently for *D. wrightii* plants?

N. attenuata and *D. wrightii* are host plants that differ in several aspects. *N. attenuata* has been shown to emit herbivore-specific feeding-damage induced volatile blends upon herbivore damage, thereby attracting predators and parasitoids (Halitschke et al. 2001, Kessler and Baldwin 2001, Allmann and Baldwin 2010). The herbivory-induced volatile emission in *D. wrightii* however, was found to be a general indicator of herbivory since emissions did not consistently differ between herbivore species (Hare and Sun 2011a). This difference in the specificity of herbivore-induced emissions might be an indicator of different plant strategies in response to herbivory by *M. sexta*. In the case of *N. attenuata* the strategic response is clearly defence. Direct defences, e.g. the production of nicotine and anti-digestive trypsin protease inhibitors, act in concert with indirect defences i.e., herbivore-induced volatile emission attracting predators, to reduce egg and larval survival, and ultimately herbivore damage (Kessler and Baldwin 2001, Kessler and Baldwin 2004, Schuman et al. 2012a). In contrast to *N. attenuata, D. wrightii* is a considerably bigger plant with large leaf surfaces. This might allow the plant a different strategy: tolerance. In the field, the biannual *D. wrightii* plants can grow to substantial size (Figure 2, introduction), whereas the annual *N. attenuata* is limited in compensating herbivory by its size. It has repeatedly been cited that a single *M. sexta* larva is able to defoliate a complete host plant (McFadden 1968). For *D. wrightii* this statement is, however, based on a rough estimate not on observation (Bronstein et al. 2009). Several studies mention the pronounced ability of *D. wrightii* to rapidly compensate for tissue loss (Bronstein et al. 2009, Reisenman et al. 2010, Reisenman et al. 2013). Thus, a damaged *D. wrightii* plant might still be evaluated as an acceptable oviposition site in terms of feeding competition.

Besides herbivory, the relationship of both host plants to *M. sexta* comprises another antagonistic perspective: adult moths constitute important pollinators for both host species. Flowers of *N. attenuata* are visited and pollinated by nocturnal *Manduca* hawk moths and diurnal hummingbirds (Aigner and Scott 2002), whereas *D. wrightii* is reported to be pollinated almost exclusively by *M. sexta* (Riffell et al. 2008b). Both species share the dilemma of necessarily attracting *M. sexta* for pollination but facing the consequences of oviposition, which often follows nectar feeding (Adler and Bronstein 2004). Due to its diurnal avian pollinators, *N. attenuata* evolved strategies to escape the herbivory-associated pollination by *M. sexta*. Upon *M. sexta* herbivory, the plant shifts flowering from dusk to dawn, the time hummingbirds occur,

and reduces the emission of a moth-attracting floral compound to avoid further feeding-related oviposition (Kessler et al. 2010). Both host species benefit from pollination by outcrossing (Kessler et al. 2008, Bronstein et al. 2009). In *D. wrightii* plants, this benefit of hawk moth pollination seems to outweigh the costs of herbivory leading to a mutualistic relationship between *D. wrightii* and *M. sexta* (Bronstein et al. 2009).

The fact that *M. sexta* moths do not generally reduce oviposition on *D. wrightii* in response to herbivory does not necessarily imply that they do not care at all. In chapter II we were able to show in field experiments that females of *M. sexta* and the congeneric *M. quinquemaculata*, which co-occur and cannot easily be differentiated in flight, indeed do differentiate during oviposition on *D. wrightii*, on the same plant. They deposited fewer eggs on leaf areas that emitted higher amounts of (*E*)-2-hexenyl acetate. The increased emission of the (*E*)-2-configured isomer of this and several other GLVs result in a specific, low ratio of (*Z*)-3/(*E*)-2-configurational isomers, which is associated with ongoing herbivore damage. Volatile emission in response to mechanical damage, in contrast, is characterised by high amounts of the (*Z*)-3-configured isomer, and a high (*Z*)-3/(*E*)-2-ratio, respectively. Thus, *D. wrightii* provides a herbivory-specific signal induced by *M. sexta* larvae themselves (chapter II). The conversion from the (*Z*)-3- to the (*E*)-2-configuration is caused by an isomerase present in the oral secretion of feeding *M. sexta* larvae (Allmann and Baldwin 2010). Therefore, this herbivory-specific signal is locally restricted to the feeding site and supposedly acts at close distance. It has been shown that a major predator of *Manduca* eggs and early stage larvae, *Geocoris* ssp uses the low (*Z*)-3/(*E*)-2-ratio in GLV emission to locate its prey (Allmann and Baldwin 2010). Hence, this ratio-coded signal enables ovipositing *Manduca* moths to locate and consequently avoid areas of high predation risk and ongoing feeding competition within a plant.

Semiochemicals identified

So far, data clearly demonstrate that plant odour mediates host choice in *M. sexta* females. We performed volatile collections and subsequent chemical analyses to examine, which features of the volatile bouquet emitted by the host plants *D. wrightii* and *N. attenuata* allow for host discrimination. When comparing the volatile profiles emitted by the two differentially preferred host species, the complexity of the host blends becomes apparent. The natural plant bouquet consists of a wide array of chemical components, with many of them being emitted only at minute amounts. We identified 45 compounds in the headspace of intact *D. wrightii* plants, and over 65 in *N. attenuata*. The two plant species share about 30 of these compounds. Nevertheless, their volatile blends are clearly discriminable by statistical analyses (chapter I). Besides qualitative differences and different ratios among the shared compounds both species

differ considerably in their total emission rate, which is about five times higher in *N. attenuata*. Hence, *M. sexta* females could potentially evaluate qualitative and quantitative characteristics of the host plant bouquet as well as signal intensity when searching for suitable host plants.

Combined, our oviposition and electrophysiological experiments showed that *M. sexta* moths, although detecting feeding-damage derived volatile emissions of both host plants, did not always discriminate between these plants behaviourally (chapter I). However, vegetative plant volatiles comprising many ubiquitous compounds were sufficient to mediate host species choice (chapter I, III). The question how insects find their specific host plant and do not accidentally target a non-host species has occupied scientific minds since a long time. Two main hypotheses arose during the last decades: insects recognise hosts via (i) volatiles that are highly specific and do not occur in unrelated species (Fraenkel 1959b) or (ii) specific ratios of volatiles, which are generally distributed among plant species (Bruce et al. 2005a). The idea of plant-taxon specific plant compounds mediating host acceptance, so-called token stimuli (Dethier 1947), got convincing support by studies from many directions, amongst others lepidopteran, coleopteran and dipteran species (Fraenkel 1959b, Schoonhoven et al. 2005). The chemicals in these studies, however acted mostly on the plant surface as feeding and oviposition stimulants. Plant species- or family-specific volatile compounds acting as attractants from a distance, have not been identified so often. The isothiocyanates, volatile products of glucosinolates and characteristic for brassicaceous plants, are well studied examples due to the considerable damage occurring on these crop plants by several pest species (Nottingham et al. 1991, Blight et al. 1995, Han et al. 2001, Ahuja et al. 2010). Several herbivores like the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae), the cabbage aphid *Brevicoryne brassicae* (Homoptera: Aphididae) and the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) respond to these compounds with high sensitivity (Nottingham et al. 1991, Blight et al. 1995, Han et al. 2001) and show attraction behaviour to them (Han et al. 2001). However, the isothiocyanates constitute a huge class of compounds (Fahey et al. 2001). Brassicaceous plants often share the same isothiocyanate-derived compounds differing only in the combination thereof, and herbivores are mostly attracted to such species-specific isothiocyanate bouquets (Nottingham et al. 1991, Blight et al. 1995, Ahuja et al. 2010). As further example of odour-based host plant orientation, in the carrot fly *Psila rosae* (Diptera: Psilidae) trans-asarone was identified as the dominant field attractant and oviposition stimulant (Guerin et al. 1983).

Experimental support for the ratio-specific host finding hypothesis, in contrast, is numerous. In a pioneering wind tunnel study Visser and Avé (1978b) manipulated the host blend of potato

plants by adding single compounds, which were known to be part of the potato volatile bouquet. While the Colorado beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) was attracted to the host blend, the behaviour changed to neutral or avoidance by adding single blend compounds with ratios among blend constituents being distorted. The authors concluded that the change in behaviour was caused by altered proportions in the combined blend and that olfactory orientation is mediated by a complex of volatiles. The attraction of the grape berry moth *Paralobesia viteana* (Lepidoptera: Tortricidae) to its natural host plant *Vitis* sp. could be mimicked by a seven-component mix containing ubiquitous plant volatiles (Cha et al. 2008). Variations in their ratio by doubling the proportion of single compounds resulted in a considerable loss of attraction (Cha et al. 2011). The black bean aphid *Aphis fabae* (Hemiptera: Aphididae) was shown to be attracted to a mix built of nine components in concentrations which were repellent to the aphids when tested singly leading to the conclusion that the same compound can act as host and non-host cue depending on the context it is perceived in (Webster et al. 2010a). A mix of three terpenoids was able to attract female grapevine moths *Lobesia botrana* as effectively as a grape cluster (Tasin et al. 2005).

For *M. sexta* it was shown that females orientate towards a blend of eight plant compounds, which were found in the headspace samples of the four host plants tomato *L. esculentum*, bell pepper *Capsicum annuum*, jimsonweed *D. wrightii* and devil's claw *P. parviflora*. None of these compounds is specific to solanaceous plants, which leads to the assumption that there is no "signature compound for host plant selection by *M. sexta*" (Fraser et al. 2003). The wind tunnel experiments we performed in chapter III support this supposition. When we presented the headspace of single host plants and a clean air control to mated *M. sexta* females, both host species *N. attenuata* and *D. wrightii* were highly preferred. When we combined both species' headspaces into a 1:1-mixture, this stimulus was not longer attractive compared to the control. This result suggests that hosts are recognised as individual species based on their odorant profile. Preferences are potentially associated with inherited or learned odour templates, and species-specific compounds or ratios are needed to facilitate discrimination. In our experiment, mixing the headspaces supposedly resulted in a new odour image, whose origin was masked by unfamiliar ratios or combinations of compounds and thus, not attractive to *M. sexta* females. Hence, semiochemicals mediating host choice between species very likely consist of both, species-specific and common volatiles, whose composition and ratio differs between host species. However, the identification of the respective odour features, i.e. compounds and their ratios, still needs to be accomplished to fully understand how plant volatiles mediate host discrimination in *M. sexta*. For the grapevine moth *L. botrana* it was recently shown that both

species-specific and common compounds are used to recognize and distinguish host species (Tasin et al. 2010).

When comparing host condition (chapter I) it has to be emphasised that herbivore-induced volatiles must act at a plant species-specific background to affect oviposition choice. The moths have been shown to reduce oviposition only in response to feeding damaged plants of *N. attenuata*, not to *D. wrightii*. Furthermore, a reduction in oviposition to *N. attenuata* was only observed when moths encountered the full authentic blend, i.e. signal of the actual feeding-damaged plant. Presentation of larval faeces, mechanically, chemically or feeding-induced plants without the larvae present did not affect oviposition (chapter I). Similar results were obtained for *D. wrightii* and *S. lycopersicum* (Reisenman et al. 2013). Interestingly, in classic field experiments the chemical induction of *N. attenuata* resulting in emission of several HIPVs was sufficient to reduce oviposition of *M. quinquemaculata* moths without an additional bouquet delivered by larvae or larval faeces (Kessler and Baldwin 2001). This discrepancy may emerge from the deficient comparability of (i) field and laboratory experiments with differing host density and movement range, (ii) naive lab-reared and experienced, wild animals and finally (iii) two congeneric but distinct species *M. sexta* and *M. quinquemaculata.*

It has been shown that the emission of herbivore-induced plant volatiles (HIPVs) as a blend repel ovipositing females of several moth species like *Heliothis virescens* (Lepidoptera: Noctuidae, De Moraes et al. 2001), *M. sexta* and congeneric *M. quinquemaculata* (Kessler and Baldwin 2001, Kessler and Baldwin 2004) and *P. viteana* (Cha et al. 2011). In chapter II we could reveal that a single compound, (*E*)-2-hexenyl acetate, presented in a low (*Z*)-3/(*E*)-2-ratio with its (*Z*)-3-configured isomer influenced oviposition choice on *D. wrightii*. The increased emission of (*E*)-2-hexenyl acetate was clearly herbivory-induced since the compound derives from the HIPV (*E*)-2-hexenal, which was converted from its configurational isomer (*Z*)-3-hexenal by an isomerase present in the oral secretions of larval *M. sexta* (Allmann and Baldwin 2010). A single HIPV, i.e. linalool, may reduce oviposition of *Manduca* moths. The full blend of *Manduca* larvae feeding upon a *N. attenuata* plant, however, was most avoided for oviposition (Kessler and Baldwin 2001). In our analyses of the *N. attenuata* volatile blend the emission of linalool was below the detection limit and single sensillum experiments did not reveal any response to this compound (chapter I). In *D. wrightii*, however, the influence of specifically (R)-(-)-linalool on *M. sexta* oviposition has been shown, but without any association to herbivory (Reisenman et al. 2010).

Perception & processing of semiochemicals

Plant odours differ among species and condition in qualitative and quantitative aspects as well as in total emission rates, see above and (Mumm and Dicke 2010). Furthermore, considerable variation within each chemical profile has been documented here and in numerous other studies (Mumm and Dicke 2010, Webster et al. 2010b, Hare and Sun 2011b). It is therefore of interest how the sensory system of an herbivorous insect evolved to accommodate variation of host plant volatiles, while allowing reliable discrimination of plants at different levels of the respective preference hierarchies. Electrophysiological experiments revealed the quality of the insect peripheral sensory system, which has been shown to be both sensitive and selective in response to odours such as pheromones (Kaissling et al. 1989) or plant volatiles (Hansson et al. 1999, Rostelien et al. 2000a, Rostelien et al. 2000b, Angioy et al. 2003, Bengtsson et al. 2011). When we applied headspace samples from *D. wrightii* and *N. attenuata* to the *M. sexta* antennal OSNs via the gas chromatograph (GC-SSR) we presented host stimuli with biologically relevant compound ratios. Under these conditions we focused on selectivity of the sensory periphery as such and the sensilla we recorded from. The periphery of *M. sexta* females responds to 120 odours, representing almost 60% of the compounds present in the host plant's volatile bouquet (chapter I). Thus, the olfactory receptors (OR) located in antennal OSNs respond to a surprisingly wide array of host volatiles.

Discrimination between host plants from the same plant family is a special challenge to the insect olfactory system, since volatile bouquets potentially overlap in composition (chapter I). We found the *M. sexta* antenna to be equipped with OSNs that by their response profiles are already sufficient to distinguish between the blends emitted by the host plants *D. wrightii* and *N. attenuata*. Using random forest analysis we could show that host blends separate well based on the emission of their compounds. This separation remained when we reduced the set of compounds to those detected by the sensilla in our SSR-experiments (chapter I). Thus, *M. sexta* females are in principle capable to discriminate hosts from a distance based on olfactory information. Our wind tunnel experiments demonstrated that the moths take advantage of this feature during host location and to establish an olfactory guided preference.

A high percentage of OSNs responded to GLVs present in the host blends such as (*Z*)-3-hexenal, (*Z*)-3-hexenol or (*Z*)-3-hexenyl acetate. Since GLVs are released by almost every green plant immediately upon damage (Gatehouse 2002, Allmann and Baldwin 2010) they constitute a commonly distributed signal. Electrophysiological studies show that many insects respond to ubiquitous plant volatiles (Bruce et al. 2005a). This non-selectivity in detecting plant volatiles is associated with the ability to evaluate ratios of host volatiles during host location and

recognition (Bruce et al. 2005a, Bruce and Pickett 2011). Instead of species-specific compounds (Dethier 1947) these insects use an odour image consisting of typically three to ten components (Bruce and Pickett 2011) of the host plant's volatile bouquet. The olfactory system recognizes the odour image or 'gestalt' (Dethier 1974) only, if these compounds are detected at the same time in adequate proportions. Several studies have shown that presenting the odour image defining compounds together results in a stronger behavioural response compared with the single compounds (Bruce and Pickett 2011). In *M. sexta*, wind tunnel experiments with a 9-component mixture derived from *D. wrightii* floral volatiles elicited robust attraction of the moths while single compounds were not attractive when presented individually (Riffell et al. 2009).

We identified both broadly and narrowly tuned antennal OSNs responding to host headspace volatiles (chapter I). OSNs responding selectively to few plant volatile compounds have been reported for *M. sexta* (Shields and Hildebrand 2000) and other insect species as well (Anderson et al. 1996, Hansson et al. 1999, Rostelien et al. 2005, Ulland et al. 2008). Specific OSNs are thought to facilitate the detection of ratios (Dejong and Visser 1988b, Bruce and Pickett 2011). In our experiments, however we found OSNs housed in sensilla with species-specific response profiles, i.e. OSN assemblies responded to compounds that were emitted only by one of the host plants. The existence of such species-specific OSNs suggests that the moth might combine two strategies during the coding of host volatile emission. A ratiometric component detects general host volatiles and their ratios via broadly tuned OSNs, while a specific component of the system detects species-discriminatory compounds. In this way, the specific component could assure host discrimination while the ratiometric part might allow flexibility against variability in host volatile emission. Correspondingly, the plasticity of the insect olfactory system established by redundancy of host volatiles was shown for other species like *P. viteana* (Cha et al. 2008) and *L. botrana* (Tasin et al. 2007). If *M. sexta,* as indicated by our results, uses ratiometric and specific components for host odour coding, the transmission of the host odour image to the central nervous system must occur in a highly combinatorial way.

Upon detection, olfactory information is clustered in the antennal lobe (AL), the first site of olfactory processing. Already here, plasticity-processing related to insect experience (Anton et al. 2011) and physiological condition such as mating status (Barrozo et al. 2011, Saveer et al. 2012) takes place via modification of olfactory processing (Anton et al. 2007). How does the olfactory system detect relevant blends among the incoming information of the plethora of odours continuously hitting the antenna? In *M. sexta* a 9-component blend derived from the

floral bouquet of *D. wrightii* was found to mediate feeding behaviour. Multi-unit recordings of *M. sexta* AL neurons revealed high synchronization of the neuronal activity, which supposedly facilitates the coding of the odour image (Riffell et al. 2009).

In chapter II we examined whether and how the olfactory system codes the larval feeding-induced change in the ratio of (*Z*)-3 and (*E*)-2-GLVs (9:1 to 1:1) emitted from *D. wrightii* plants. Using functional imaging, we found two OSN populations responding specifically to either the (*Z*)-3- or the (*E*)-2-configuration of hexenyl acetate. At the doses tested, a concentration dependence was only found for the (*E*)-2-specific region in the *M. sexta* AL, suggesting that the difference between the two (*Z*)-3/(*E*)-2-ratios is perceived mainly via (*E*)-2-hexenyl acetate. Since we only observed the OSN input to the *M. sexta* AL we can only speculate about further processing of the incoming odour information. From physiological studies on blend interactions in the *M. sexta* AL we know that blend processing is highly combinatorial due to non-linear interactions mediated by local interneurons (Kuebler et al. 2011, Kuebler et al. 2012). For the AL input, however, odour blends are represented as a linear sum of their single components (Silbering et al. 2008, Deisig et al. 2010, Kuebler et al. 2012). In contrast, in *L. decemlineata* (Dejong and Visser 1988b) found that suppression dominated the coding of binary GLV mixtures in specialized OSNs, leading to the assumption that information about host volatiles is conveyed via two channels to the AL. Broadly tuned OSNs responding additively to binary mixtures convey quantitative information, while specialised OSNs code quality i.e. the deviation of the host-relevant ratio (Dejong and Visser 1988b). Consequently, specialised local interneurons in the AL did not respond to a host leaf extract, whereas the neurons responding to all tested GLVs non-specifically showed strong responses (Dejong and Visser 1988a).

With this thesis I demonstrate that olfaction plays a central role for egg laying *M. sexta* females. From a distance females detect host plant volatiles and decide whether to approach a potential oviposition site or not. Host volatile bouquets contain information about plant species and condition and the peripheral olfactory sense of *M. sexta* is equipped to discriminate between them. However, oviposition depends on species as well as context. They reflect particular characteristics of the herbivore-host relationship. While conspecific herbivory on *N. attenuata* results in reduced oviposition on the plant*, D. wrightii* plants are still accepted: however, the actual oviposition site on the plant is selected most probably to avoid competition and/or predation risks. By doing so, *M. sexta* exploits *D. wrightii* as resource for its offspring, which is known to quickly refoliate after feeding damage. The discriminating oviposition behaviour

depending on host species and condition is enabled by a highly sophisticated olfactory system, which reliably recognises relevant volatile signals such as complex host blends or the ratio of (*E*)-2- and (*Z*)-3-configurations of GLV compounds. The system utilises a combination of broadly and narrowly tuned OSNs to code encountered emissions of host volatiles. During host recognition, the detected volatile profile is evaluated against the innate or learned specific olfactory 'gestalt' of the host plant, which depends on host blend composition.

A core challenge for olfaction research will be to characterise the features that make up the olfactory 'gestalt' under real host choice conditions, and to understand how flexible the system responds to the important variation found in plant volatile emissions.

SUMMARY

To provide favourable and secure conditions benefiting the offspring is a ubiquitous struggle among the world's creatures. For herbivorous insects that do not support their offspring after egg deposition the choice of host plant plays an essential role to ensure reproductive success. Various physiological and ecological factors like nutritional quality, palatability, and shelter from enemies influence the performance of herbivores. An egg laying female needs to take these criteria into account, and the influence of these factors on host plant choice has been studied intensely. For the tobacco hawk moth *Manduca sexta* (Lepidoptera: Sphingidae), a model in insect olfaction research, host and food search as well as oviposition preferences have been examined. Females exhibit preference hierarchies between species as well as for different plant conditions within species. Among the chemical senses, olfaction plays a key role in host location for many insects. This is especially true for night-active insects and may, thus be expected for *M. sexta* as well. However, still little is known about the impact of olfaction on the choice among available host plants. Different stimuli act on *M. sexta* during the behavioural sequences that result in oviposition, and it is still unsolved, to which extend host choice is mediated by visual or olfactory cues from a distance or by gustatory or tactile cues during plant contact. The question arises, how host preferences are established at the sensory, processing and behavioural level. Which host cues adumbrate reproductive success towards an ovipositing insect?

This thesis focuses on the host choice of the oligophagous herbivore *M. sexta* and aims at elucidating the role of host plant derived volatiles in this process by applying behavioural, analytical, electrophysiological and Ca^{+}-imaging methods. The data presented here give insights into egg laying preferences, chemical identity of olfactory cues, and detection and processing of choice-mediating semiochemicals, i.e. volatile compounds that convey information between host plant and insect.

The data presented here demonstrate that vegetative plant volatiles suffice to mediate the choice of host species. The species-specific oviposition preferences that *M. sexta* females exhibited (chapter I) are consistent with host preferences shown in wind tunnel experiments that exclusively presented plant headspace volatiles as host derived cues (chapter III). Thus, olfaction is essential for *M. sexta* females, to locate, evaluate and accept host plants for egg deposition. *M. sexta* females perform their host choice using a host-specific olfactory 'gestalt',

which is mainly dependent on blend composition but can be influenced by signal intensity, i.e. total volatile concentration as well (chapter III).
The often reported avoidance of feeding-damaged host plants by egg laying insects is commonly associated to predation risk for the offspring on plants that persuasively advertise the presence of the herbivore to a third trophic level. However, in *M. sexta* the preference for intact over feeding damaged host plants is species-specific (chapter I) thereby reflecting particular characteristics of the respective herbivore-host relationship. While herbivory on *Nicotiana attenuata* results in reduced oviposition on the plant*, Datura wrightii* plants are still accepted though vigilantly assessed before egg deposition: The actual oviposition site on the plant is selected most probably to avoid larval feeding competition and/or predation risks (chapter II). By doing so, egg laying *M. sexta* exploit *D. wrightii* plants as a resource for its offspring, which in contrast to *N. attenuata* is known to quickly refoliate after feeding damage.

The discriminating oviposition behaviour depending on host species and condition is facilitated by a highly sophisticated olfactory system, which reliably recognizes relevant volatile signals such as complex host blends or in the case of feeding-damaged *D. wrightii* the ratio of (*E*)-2- and (*Z*)-3-configuration of green leaf volatile compounds. Based on discrete chemical profiles, the olfactory system of *M. sexta* is able to discriminate host species and condition via broadly and narrowly tuned olfactory sensory neurons. Some sensory neurons display plant species-specific responses giving rise to overlapping but still distinct activity pattern (chapter I). Wind tunnel experiments allow for the conclusion that during host recognition, the detected volatile profile is evaluated against the innate or learned specific olfactory 'gestalt' of the host plant, which depends on host blend composition.

M. sexta females navigate through a complex chemical world. Host plants, potentially affected by plant-feeding herbivores, deliver messages enrobed in a volatile bouquet to the moth. An elaborate olfactory system, which is extremely well tuned to extract information from such volatile signals, ensures the selection of host plants that will most probably provide reproductive success to the ovipositing female.

ZUSAMMENFASSUNG

Die nützliche Versorgung der eigenen Nachkommen mit günstigen sowie sicheren Bedingungen ist ein allgegenwärtiger Kampf, der von allen Kreaturen der Erde geführt wird. Für pflanzenfressende Insekten, die ihren Nachwuchs nach der Eiablage nicht weiter unterstützen, ist die Wahl der Wirtspflanze in Bezug auf den reproduktiven Erfolg von grundlegender Bedeutung. Diverse physiologische und ökologische Faktoren wie die Nahrungsqualität und Genießbarkeit der Wirtspflanze sowie der Schutz vor Fressfeinden beeinflussen die Leistung von Herbivoren, die sich von Wirtspflanzen ernähren. Für eine optimale Wahl sollten Insekten diese Kriterien bei der Eiablage berücksichtigen. beachtet werden. Der Einfluss jener Faktoren auf die Wirtswahl wurde bereits intensiv erforscht. Für den Tabakschwärmer *Manduca sexta* (Lepidoptera: Sphingidae) - ein Modellorganismus für den Geruchssinn bei Insekten – sind Nahrungs- und Wirtssuche sowie Eiablagepräferenzen gut untersucht. Weibchen zeigen Präferenzhierarchien, sowohl zwischen Pflanzenarten als auch für Zustände von Pflanzen einer Art. Unter den chemischen Sinnen nimmt der Geruchssinn eine Schlüsselrolle bei der Wirtssuche von Insekten ein. Dies gilt insbesondere für nachtaktive Insekten, und ist daher auch für *M. sexta* zu erwarten. Dennoch ist die Bedeutung des Geruchssinns bei der Auswahl aus vorhandenen Wirtspflanzen weitestgehend ungeklärt. Verschiedene Reize wirken auf *M. sexta* während einzelner Sequenzen des Eiablageverhaltens ein. Es ist noch immer unklar, ob die Wirtswahl mittels visueller oder olfaktorischer (geruchlicher) Reize aus der Distanz oder durch geschmackliche oder taktile Reize während des Kontakts zur Pflanze entscheidend vermittelt wird. Es stellt sich daher die Frage, wie Wirtspräferenzen auf sensorischer, verarbeitender und verhaltensrelevanter Ebene gefestigt werden. Welche Signale der Wirtpflanze deuten dem Weibchen bei der Eiablage reproduktiven Erfolg an?

Diese Doktorarbeit beschäftigt sich mit der Wirtspflanzenwahl des oligophagen Herbivors *M. sexta* mit dem Ziel, die Rolle der wirtseigenen Pflanzenduftstoffe in diesem Prozess zu erhellen. Dabei werden Verhaltensversuche, chemisch-analytische, elektrophysiologische und optophysiologische Methoden verwendet. Die Arbeit zeigt Erkenntnisse zu Eiablagepräferenzen, zur chemischen Identität von olfaktorischen Reizen sowie zur Wahrnehmung und Verarbeitung von wahlentscheidenden Semiochemikalien, d.h. volatilen Botenstoffen, welche Informationen zwischen Wirtspflanze und Insekt übermitteln.

Die hier vorgestellte Arbeit demonstriert, dass vegetative Pflanzenduftstoffe ausreichen, um die Wahl der Wirtsart herbeizuführen. Die artspezifischen Eiablagepräferenzen von *M. sexta* (Kapitel

I) stimmen mit Wirtspräferenzen überein, die in Windkanal-Experimenten unter Ausschluss aller wirtsrelevanten Sinnesreize außer den Pflanzenduftstoffen beobachtet wurden (Kapitel III). Demzufolge ist der Geruchssinn essentiell für Weibchen von *M. sexta*, um Wirtspflanzen zu finden, zu bewerten und für die Eiablage zu akzeptieren. Für die Wirtswahl nutzen die Weibchen dabei eine verinnerlichte, artspezifische olfaktorische „Gestalt", welche überwiegend durch die Komposition des Wirtsduftes geprägt wird, jedoch auch von der Intensität des Duftsignals, also der absoluten Duftkonzentration Beeinflussung erfährt (Kapitel III).

Die oft berichtete Vermeidung von fraßgeschädigten Wirtspflanzen durch Insekten bei der Eiablage wird gemeinhin mit einem erhöhten Prädationsrisiko für die Nachkommen auf jenen Pflanzen in Verbindung gebracht. Denn Pflanzen zeigen zumeist die Anwesenheit von Herbivoren verführerisch duftend einer dritten trophischen Ebene von Fressfeinden an. Im Fall von *M. sexta* jedoch ist die Bevorzugung intakter gegenüber fraßgeschädigter Pflanzen wirtsartspezifisch (Kapitel I), wodurch sich bestimmte Eigenschaften der jeweiligen Herbivor-Pflanzen-Beziehung widerspiegeln. Während Herbivorie bei *Nicotiana attenuata* zu einer reduzierten Eiablage führt, wird *Datura wrightii* weiterhin für die Eiablage nach aufmerksamer Prüfung akzeptiert: Die tatsächliche Eiablagestelle an der Pflanze wird höchstwahrscheinlich so gewählt, dass Fraßkonkurrenz seitens der Raupen und/oder Prädationsrisiko vermieden werden (Kapitel II). Dies erlaubt eiablegenden Weibchen *D. wrightii* Pflanzen als Ressource für ihren Nachwuchs auszuschöpfen, welche im Gegensatz zu *N. attenuata* für schnelles Austreiben neuer Blätter nach Fraßschaden bekannt sind.

Das kritische Eiablageverhalten bezüglich Wirtsart und Zustand wird durch ein hochentwickeltes olfaktorisches System ermöglicht. Es erkennt verlässlich relevante Duftsignale wie zum Beispiel komplexe Wirtsduftmischungen oder im Fall der fraßgeschädigten *D. wrightii* das Verhältnis von (*E*)-2- und (*Z*)-3-Konfigurationen von grünen Blattduftstoffen. Basierend auf chemisch diskreten Profilen kann das olfaktorische System von *M. sexta* Wirtsart und Wirtszustand mithilfe unspezifischer sowie auf die Detektion einzelner oder weniger Verbindungen spezialisierter olfaktorischer Rezeptorneurone unterscheiden. Einige Rezeptorneurone zeigen artspezifische Antworten und erzeugen damit überlappende und doch eindeutige Aktivitätsmuster (Kapitel I). Windkanal-Experimente lassen die Schlussfolgerung zu, dass während der Wirtserkennung das entdeckte Duftprofil gegen eine angeborene oder erlernte spezifische „Duftgestalt" der Wirtsart abgeglichen wird.

Weibchen von *M. sexta* navigieren in einer durch und durch chemischen Welt. Wirtspflanzen, die potenziell von pflanzenfressenden Herbivoren befallen sind, überbringen der Motte duftend

verpackte Nachrichten. Ein ausgeklügeltes Geruchssystem, das perfekt auf die Gewinnung von Informationen aus Duftsignalen ausgelegt ist, gewährleistet die Wahl jener Wirtspflanzen, die dem eiablegenden Weibchen den wahrscheinlichsten reproduktiven Erfolg bieten.

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DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

Jena, March 26, 2013